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Cyanobacteria

Exploring Their Role in Energy,
Environment and Industry

*Edited by Ihana Aguiar Severo
and Mariane Bittencourt Fagundes*



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Meet the editors



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Preface

Cyanobacteria are among the most ancient and influential organisms on Earth, playing a fundamental role in shaping the planet's atmosphere, ecosystems, and biological evolution. Today, these microorganisms are gaining renewed attention for their exceptional potential to address contemporary challenges in energy production, environmental sustainability, and industrial biotechnology.

This edited volume, *Cyanobacteria – Exploring Their Role in Energy, Environment and Industry*, brings together contributions from experts across disciplines to provide a comprehensive overview of cyanobacterial research, ranging from fundamental biological processes to selected applied perspectives. The book covers key topics, including i) an introduction to cyanobacteria, ii) biological aspects of cyanobacteria for industrial applications, iii) structural and functional roles of inorganic cofactors of photosystem II, iv) genetic modification strategies, v) algae-based biorefineries, vi) commercial potential of cyanobacteria through biochemical and taxonomical analysis, and vii) ecological dynamics influencing cyanobacterial dominance in natural systems.

This volume emphasizes foundational concepts, biological mechanisms, and representative case studies that illustrate the diverse roles of cyanobacteria in natural and engineered systems. The chapters provide a solid scientific basis for understanding cyanobacterial physiology, genetics, ecological dynamics, and emerging applications, serving as a reference for readers seeking a comprehensive overview of the field. This book is intended for researchers, graduate students, and professionals interested in cyanobacteria from biological, environmental, and applied science perspectives.

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Introductory Chapter: Cyanobacteria – Exploring Their Role in Energy, Environment and Industry

Íhana Aguiar Severo and Mariane Bittencourt Fagundes

1. Overview of cyanobacteria: ancient microorganisms with modern relevance

Cyanobacteria are among the most ancient life forms on Earth, with fossil records dating back more than 3.5 billion years. Through oxygenic photosynthesis, these microorganisms fundamentally transformed the planet's atmosphere, paving the way for the evolution of aerobic life and shaping global biogeochemical cycles. Despite their prokaryotic cellular organization, cyanobacteria exhibit remarkable metabolic diversity and ecological adaptability, thriving in freshwater, marine, and terrestrial ecosystems worldwide. Their evolutionary success and versatility have attracted increasing attention for biotechnological applications focused on sustainability and resource efficiency [1, 2].

Unlike eukaryotic microalgae, cyanobacteria possess a prokaryotic cell structure and, in many species, the ability to fix atmospheric nitrogen [3]. These characteristics make them particularly attractive as biological factories capable of generating a wide range of bio-based products using sunlight, water, and CO₂ as the main resources. To date, more than 2,000 bioactive metabolites have been identified from cyanobacterial species, underscoring their biochemical richness and industrial relevance [4].

From an evolutionary perspective, this versatility is strongly linked to the cyanobacterial pangenome, which encompasses the extensive and diverse gene pool distributed across different lineages. Rather than relying solely on gradual mutations, cyanobacteria rapidly adapt to environmental changes through genome fluidity and horizontal gene transfer, particularly within heterogeneous genomic regions [5]. Mechanisms such as transduction, conjugation, and transformation have operated throughout their evolutionary history, enabling gene exchange across lineages for billions of years [6].

As a result, cyanobacterial genomes often display a mosaic architecture, where genetic innovation arises from both inherited traits and acquired functions. This evolutionary resilience has allowed cyanobacteria to survive major planetary transitions, including the Great Oxygenation Event and multiple mass extinction episodes, while continuously expanding into new ecological niches [7].

2. Cyanobacteria in the context of sustainability and the bioeconomy

As the global community transitions toward a low-carbon future, cyanobacteria have emerged as promising contributors to the bioeconomy. Their ability to produce bioenergy and bioproducts without competing with food crops offers a sustainable path for circular resource utilization. Cyanobacterial systems can be integrated into biorefineries that couple waste valorization with biomass generation, contributing to carbon capture and nutrient recycling. The cultivation of microalgae and cyanobacteria using wastewater and digestate is central to developing circular biorefineries, as emphasized by recent reviews [8–12].

The metabolic flexibility of cyanobacteria enables the synthesis of biofuels such as biohydrogen, biodiesel precursors, and biogas [13, 14]. Moreover, they produce valuable compounds like pigments (phycocyanin, chlorophyll, carotenoids) [15–17], bioplastics (polyhydroxyalkanoates) [18], biofertilizers [19], pharmaceuticals [20], and biomedical applications [21]. In addition to well-known pigments and polymers, cyanobacteria synthesize lipid-derived metabolites such as sterols and squalene, which play important roles in membrane structure, cellular adaptation, and stress tolerance. These compounds have been identified and produced in strains such as *Phormidium autumnale*, highlighting their potential as high-value products for nutraceutical, pharmaceutical, and biorefinery applications within sustainable cyanobacteria-based systems [22–24]. All of these applications align with circular economy principles, converting waste and emissions into renewable resources while reducing environmental impacts [25].

3. Environmental implications and biotechnological challenges

Cyanobacteria play a dual role in environmental systems: they are vital for ecosystem balance and carbon cycling, but they can also cause ecological concerns such as harmful algal blooms and toxin production [26–28]. Understanding and managing these dualities is central to sustainable biotechnological development. Advances in molecular biology, genomics, and environmental engineering now enable precise control over cyanobacterial metabolism and growth conditions [29–31].

Cultivation technologies have evolved from open ponds to closed photobioreactors, improving process control and product quality. However, challenges remain in large-scale cultivation, including contamination risks, harvesting efficiency, and the optimization of light and nutrient supply [32]. Future research aims to enhance strain robustness and productivity through synthetic biology and metabolic engineering.

4. Industrial and technological advances

Recent developments in biotechnology have expanded cyanobacteria's industrial applications. They are being engineered to function as biofactories for pigments, antioxidants, amino acids, and bioactive metabolites [33–37]. Their

capacity to fix nitrogen naturally also makes them suitable for sustainable agriculture and biofertilizer production. In addition, their use as feedstock for bio-based materials and biodegradable polymers positions them as a strategic platform for replacing petroleum-based products [38].

Integration with industrial systems is progressing through biorefineries and wastewater treatment plants, where cyanobacteria provide multiple benefits: bioremediation, biomass recovery, and production of high-value compounds [38]. The potential for coupling cyanobacterial processes with renewable energy systems, such as solar-driven photobioreactors, further strengthens their industrial appeal.

5. Future perspectives

The next frontier for cyanobacterial biotechnology lies in digitalization, automation, and predictive modeling of bioprocesses. Combining artificial intelligence with omics-based data can accelerate strain selection, optimize

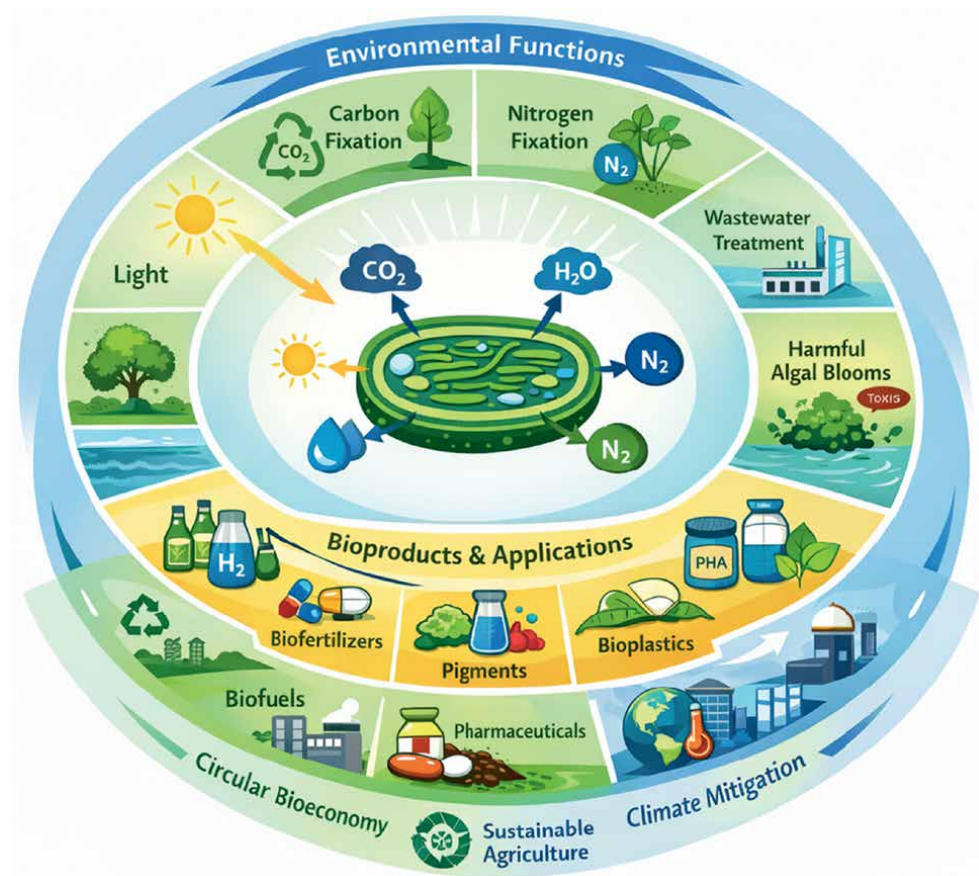


Figure 1.
 Multifunctional roles of cyanobacteria across evolutionary, environmental, and industrial dimensions (prepared using an AI tool).

metabolic fluxes, and enable continuous monitoring of large-scale systems. Furthermore, hybrid systems integrating cyanobacteria with other microorganisms are emerging as powerful platforms for multi-product biomanufacturing.

In the context of global sustainability, cyanobacteria contribute directly to several United Nations Sustainable Development Goals (SDGs), including clean energy (SDG 7), climate action (SDG 13), and responsible production and consumption (SDG 12). Their versatility, scalability, and environmental benefits make them key biological agents in building a green industrial future. **Figure 1** provides an overview of the roles of cyanobacteria across evolutionary, environmental, and industrial dimensions.

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Cyanobacteria: A Biological Chassis for Industrial Applications

Bharat Kumar Majhi

Abstract

Cyanobacteria are a diverse group of prokaryotic organisms that engage in oxygenic photosynthesis. They have served as model organisms for a wide range of photobiochemical research. Recently, they have received significant attention as a potential host for various industrial and agricultural applications due to their unique physio-biochemical properties. Their ability to withstand harsh environmental conditions and internal biochemical changes, along with their faster growth, minimal growth requirements, inherent double homologous recombination, and uptake of exogenous deoxyribonucleic acid (DNA) without the use of external forces, makes them a superior candidate for numerous applications. They have been utilized as hosts to manufacture a wide range of industrial products, including biofuel and bioplastic. Furthermore, cyanobacteria's nitrogen-fixing ability enables them to be used as a biofertilizer in sustainable agriculture. Additionally, their ability to scavenge metals from the environment makes them an excellent candidate for bioremediation. This book chapter will explore cyanobacterial applications in biofuel and bioplastic production, as well as bioremediation and sustainable agriculture. It will also highlight their distinct physiological characteristics that differentiate them from other photoautotrophs.

Keywords: cyanobacteria, biofuel, bioplastic, bioremediation, biofertilizer

1. Introduction

Advanced recombinant-DNA technology and synthetic biology have been effective tools for creating genetically modified organisms capable of producing a wide variety of industrial products [1, 2]. Numerous native organisms, including mammals, yeast, *Escherichia coli*, plants, algae, and cyanobacteria, have been genetically altered and transformed into biofactories capable of producing valuable chemicals [3]. Genetically modified host organisms are critical to the current bio-based product generation process. Although various organisms are capable of producing valuable industrial products, growth rate, biomass yield, contamination risks, environmental constraints, production cost, and yield, all play important roles in selecting the right host for biomanufacturing. Mammalian cells are heavily used in a variety of applications; however, contamination risks and processing costs pose significant challenges to bioproduct generation [4]. In contrast, fermentative

organisms such as yeast and *E. coli* offer numerous benefits as hosts, including faster growth, higher product yields, and greater resistance to contamination [5]. Despite their many advantages, using fermentative organisms as hosts to produce biochemicals incurs high production costs. Their growth is dependent on external energy sources, including expensive growth media. Furthermore, they are prone to contamination due to their nutrient-rich growth media [5].

To alleviate the aforementioned challenges, photosynthetic organisms have recently been considered as potential hosts for biomanufacturing [6–8]. In addition to being hosts for bioproduct generation, they have also been used in a variety of applications, such as bioremediation and sustainable agriculture, due to their unique physiological properties [9]. They do not require expensive growth media for cultivation, as they are photoautotrophs and synthesize organic carbon from atmospheric carbon dioxide (CO₂) using sunlight and water, which they use for their growth. Moreover, they are resistant to a wide range of pathogens, including zoonotic contaminants. However, photosynthetic organisms, such as plants and algae, have delayed physiological growth, a complex genetic reconstruction process, and low yield, making them less desirable as hosts for bioproduct generation and other applications [10, 11]. Cyanobacteria, on the other hand, have numerous advantages as hosts compared to other host species [12].

Cyanobacteria are ancient photoautotrophic organisms that have the ability to convert CO₂ to organic carbon through photosynthesis [13]. They are single-celled prokaryotic organisms that are better suited for genetic modifications due to their inherent ability to uptake exogenous DNA into the cell and integrate it into the genome through direct homologous recombination [14]. In addition, they grow faster compared to other photoautotrophs and have minimal growth requirements. They can be grown in wastewater, which can reduce cultivation costs [15]. Unlike plants, they do not require arable land to grow. They can be grown outside in low-cost open ponds or indoors in a photobioreactor with a constant light supply [16]. They also fix atmospheric nitrogen and store it in the soil, which promotes plant growth. Moreover, they are natural scavengers [9]. They uptake various toxic pollutants from their surroundings and keep the environment clean. Due to their unique inherent physiological properties, they have been heavily used in numerous applications, including the generation of biofuel and bioplastic, as biofertilizers in sustainable agriculture, and as bioremediation agents for removing toxic pollutants from the environment [9, 12]. This chapter emphasizes the use of cyanobacteria in biofuel and bioplastic production, as well as sustainable agriculture and bioremediation.

2. Cyanobacterial applications

Cyanobacteria have been widely used as model organisms in photosynthesis research [17]. Their remarkable physiological properties have sparked widespread interest in the scientific community. In addition to their involvement in photosynthesis research, they have recently been used in a wide variety of biotechnological applications. Cyanobacteria have been transformed into a biological chassis for numerous industrial and agricultural products, including therapeutic proteins, bioplastics, biofuels, biofertilizers, and remediation agents [9, 12, 18–20] (**Figure 1**).

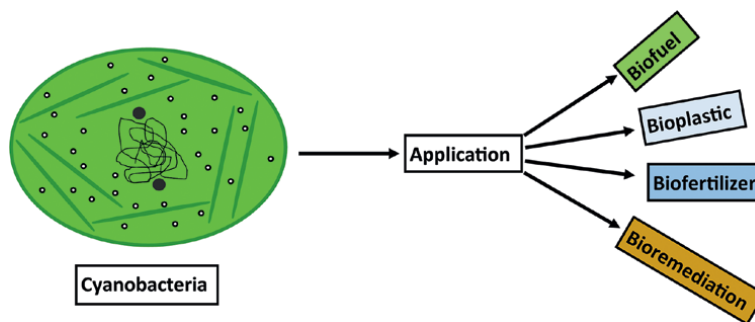


Figure 1.
 Overview of cyanobacterial applications.

2.1 Biofuel

Biofuels are renewable green energy sources derived from the biomass of photosynthetic and nonphotosynthetic organisms [21]. Photosynthetic organisms are the largest contributors to biofuel production. Photosynthetic organisms, such as plants, algae, and cyanobacteria, have been used as hosts to produce biofuel as an alternative to fossil fuels [21]. Biofuel has numerous advantages, including lower greenhouse gas emissions, eco-friendliness, and the release of less toxic gases into the environment when burned [22]. Plants, in particular, are heavily used in biofuel production; however, producing biofuel from plants poses numerous challenges, including low yield, time-consuming downstream processing, and high production costs [23]. To alleviate these issues and make biofuel production simpler, more time-efficient, and cost-effective, cyanobacteria have been used as an alternative host [12]. Cyanobacteria's unique physiological properties, such as faster growth, a simple genetic modification process, and economical cultivation methods, make them an ideal host for biofuel production [12]. Several cyanobacterial species, such as *Synechocystis* sp. PCC 6803 [24], *Synechococcus elongatus* PCC 7942, *Synechococcus* sp. PCC 7002 [25, 26], *Nostoc* PCC 7120 [27], *Anabaena* sp. PCC 7120 [28, 29], *Synechococcus* sp. PCC 11901 [30], *Synechococcus elongatus* UTEX 2973 [31, 32], *Synechococcus elongatus* PCC 6301 [33], *Anabaena variabilis* ATCC 29413 [34], *Cyanothece* sp. PCC 7425 [35], and *Cyanothece* sp. ATCC 51142 [36] has been used to produce a wide variety of biofuels, including isoprene, free fatty acids, hydrogen, n-alkanes, ethylene, 2-methyl-1-butanol, 3-methyl-1-butanol, isobutanol, ethanol, limonene, and β -phellandrene (**Table 1**).

Cyanobacteria naturally produce some biofuels, such as ethanol, hydrogen, and free fatty acids, albeit in trace amounts. However, the majority of cyanobacteria lack specific enzymes needed to synthesize biofuels [37]. As a result, cyanobacteria have been genetically and metabolically modified to incorporate heterologous enzymes or entire metabolic pathways specific to biofuel production within the cells [38–40]. The necessary modifications enabled biofuel accumulation in the cells [12]; however, the rates of biofuel production are lower compared to other host species, such as yeast and *E. coli* [41–44]. The limited availability of precursor molecules is the bottleneck contributing to the low rate of biofuel production. To address this issue and increase biofuel production, cyanobacteria have been modified to rewire the carbon flux within the cell, thereby increasing the supply of precursors [45–48]. The

Product	Yield	Strain	References
Isoprene	1.14–3.54 $\mu\text{mol g}^{-1} \text{dcw h}^{-1}$	<i>Synechocystis</i> sp. PCC 6803	[49]
	2.80–13.35 $\mu\text{g g}^{-1} \text{dcw h}^{-1}$	<i>Synechococcus elongatus</i> UTEX 2973	[50]
	1.7–46.0 $\mu\text{g L}^{-1} \text{h}^{-1}$	<i>Synechocystis</i> sp. PCC 6803	[51]
	1.1–5.1 $\mu\text{g L}^{-1} \text{h}^{-1}$	<i>Synechocystis</i> sp. PCC 6803	[52]
	4.26 $\text{mg L}^{-1} \text{h}^{-1}$	<i>Synechococcus elongatus</i> PCC 7942	[53]
Free fatty acids	40% dcw	<i>Synechocystis</i> sp. PCC 6803	[54]
	35.8–45.7 $\text{mg L}^{-1} \text{d}^{-1}$	<i>Synechocystis</i> sp. PCC 6803	[55]
	0.12 $\text{mmol L}^{-1} \text{OD}_{730}^{-1}$	<i>Synechococcus</i> sp. PCC 11901	[30]
	0.05 $\text{mmol L}^{-1} \text{OD}_{730}^{-1}$	<i>Synechococcus</i> sp. PCC 7002	
Hydrogen	32–546 $\mu\text{mol H}_2$	<i>Synechocystis</i> sp. PCC 6803	[56]
	132.3 $\mu\text{mol H}_2 \text{mg Chl a}^{-1} \text{h}^{-1}$	<i>Dolichospermum</i> sp. IPPAS B-1213	[57]
	2–23 $\text{nmol H}_2 \text{mol Chl a}^{-1} \text{h}^{-1}$	<i>Synechocystis</i> sp. PCC 6714	[58]
	7–23 $\text{nmol H}_2 \text{mol Chl a}^{-1} \text{h}^{-1}$	<i>Synechococcus elongatus</i> PCC 7942 PAMCOD	
n-alkanes	4–27 $\text{mg g}^{-1} \text{dcw}$	<i>Picosynechococcus</i> sp. strain PCC 7002	[59]
	0.0000007 $\mu\text{g per cell}$	<i>Synechocystis</i> PCC 6803	[60]
	0.15–0.17% dcw	<i>Synechococcus</i> sp. PCC 7002	[61]
	0.7–1.5 $\text{mol mol Chl a}^{-1}$	<i>Synechocystis</i> PCC 6803	[62]
	0.43 $\mu\text{g mg}^{-1} \text{dcw}$	<i>Oscillatoria</i> CCC 305	[63]
	0.13 $\mu\text{g mg}^{-1} \text{dcw}$	<i>Oscillatoria formosa</i> BDU 30603	
	300–800 $\mu\text{g g}^{-1} \text{dcw}$	<i>Synechococcus elongatus</i> PCC 7942	[64]
	600–1,200 $\mu\text{g g}^{-1} \text{dcw}$	<i>Anabaena</i> sp. PCC 7120	
Ethylene	750–1,600 $\mu\text{g g}^{-1} \text{dcw}$	<i>Anabaena halophytica</i>	
	2–16 $\mu\text{g mL}^{-1} \text{OD}_{730}^{-1} \text{d}^{-1}$	<i>Synechocystis</i> PCC 6803	[65]
	0–30 $\mu\text{mol L}^{-1} \text{h}^{-1}$	<i>Synechococcus</i> PCC 7942	[66]
	6.4–19.2 $\mu\text{g mL}^{-1} \text{OD}_{730}^{-1} \text{d}^{-1}$	<i>Synechocystis</i> PCC 6803	[67]
	338.26 $\mu\text{mol g}^{-1} \text{dcw h}^{-1}$	<i>Synechococcus elongatus</i> PCC 11801	[68]
	174–383 $\mu\text{mol mg}^{-1} \text{Chl a}$	<i>Synechocystis</i> sp. PCC 6803	[69]
Isobutanol	140 $\mu\text{L L}^{-1} \text{OD}_{750}^{-1} \text{h}^{-1}$	<i>Synechococcus elongatus</i> PCC 7942	[70]
	38 $\text{mg L}^{-1} \text{OD}_{730}^{-1}$	<i>Synechocystis</i> sp. PCC 6803	[71]
3-methyl-1-butanol	12 $\text{mg L}^{-1} \text{OD}_{730}^{-1}$		
Isobutanol	27.1–29.8 $\text{mg L}^{-1} \text{d}^{-1}$	<i>Synechocystis</i> sp. PCC 6803	[72]
3-methyl-1-butanol	7.3–9.5 $\text{mg L}^{-1} \text{d}^{-1}$		
Isobutanol	302 $\text{mg L}^{-1} \text{d}^{-1}$	<i>Synechocystis</i> PCC 6803	[73]
Ethanol	172.6 $\text{mg g}^{-1} \text{EPS}$	Atacama cyanobacteria	[74]
	2.11–2.43 g L^{-1}	<i>Synechocystis</i> sp. PCC 6803	[75]
	1.2 g L^{-1}	<i>Synechocystis</i> PCC 6803	[76]
	248 $\text{mg L}^{-1} \text{d}^{-1}$	<i>Synechocystis</i> sp. PCC 6803	[77]
	0.25% (v/v)	<i>Synechococcus</i> sp. PCC 7002	[78]

Product	Yield	Strain	References
Limonene	8.2 mg L ⁻¹ d ⁻¹	<i>Synechococcus elongatus</i> UTEX 2973	[79]
	4.3 mg L ⁻¹ d ⁻¹	<i>Synechococcus elongatus</i> PCC 7942	[80]
	0.027 mg L ⁻¹ d ⁻¹	<i>Synechocystis</i> PCC 6803	[81]
	0.042 mg L ⁻¹ d ⁻¹	<i>Synechococcus</i> PCC 7002	
	4.2 mg L ⁻¹ d ⁻¹	<i>Synechococcus elongatus</i> PCC 7942	[82]
	0.95 mg L ⁻¹ d ⁻¹	<i>Synechocystis</i> sp. PCC 6803	[83]
β-phellandrene	1.42–5.95 mg g ⁻¹ dcw	<i>Synechocystis</i> sp. PCC 6803	[84]
	3.2 mg g ⁻¹ dcw	<i>Synechocystis</i> sp. PCC 6803	[85]
	1 μg L ⁻¹ h ⁻¹	<i>Synechocystis</i> sp. PCC 6803	[86]

dcw: dry cell weight; d: day; L: liter; h: hour; EPS: exopolysaccharide; OD: optical density; Chl: chlorophyll

Table 1.
 List of cyanobacteria used in biofuel production.

increased amount of precursors within the cells enhanced biofuel production. The biofuel production rate ranges between 2.4 mg and 2.43 g L⁻¹ [12]. Although genetic and metabolic modifications enabled biofuel synthesis in the cell, production rates, cytotoxicity from overproduction of certain biofuels, and low biomass yield remain the biggest challenges in cyanobacterial biofuel production, impeding its commercial success [45–48]. These challenges must be overcome in order to make cyanobacterial biofuel production cost-effective and profitable on a large scale.

2.2 Bioplastic

Every day, humans and other organisms come into contact with plastics, either directly or indirectly. Plastics are used in a variety of applications, ranging from household items to medical devices. Polymers, particularly petrochemicals, are used in the polymerization process to create plastics [87, 88]. Petrochemical-based plastic production is expensive in terms of both money and natural resources. Furthermore, plastics are nonbiodegradable, posing a significant risk of plastic pollution. Plastic pollution is a major global concern because it harms both terrestrial and aquatic organisms [89, 90]. Therefore, it is very important to find an alternative to nondegradable plastics in order to prevent plastic pollution. Bio-based plastics have emerged as a viable alternative to nonbiodegradable plastics in recent decades [91]. Bioplastics are biodegradable and environmentally friendly because they are partially or entirely made up of biological materials. These are divided into different categories based on their biological source, including starch-based, lignin-based, protein-based, chitin-based, and aliphatic-polyester-based bioplastics [92]. Cyanobacteria have recently emerged as a potential candidate for bioplastic production [93, 94] (Table 2). They naturally produce a variety of chemicals that can be used to make bioplastics, or they can be genetically modified to produce precursors that can be used in bioplastic production [95, 96]. For example, polyhydroxyalkanoates (PHAs) are lipidic biopolymers that serve as a major precursor for bio-based plastics production [96]. Polyhydroxybutyrate (PHB), a type of PHA, is a major component of biodegradable plastics produced by various cyanobacterial species [97]. The biosynthetic pathway for PHB production in cyanobacteria involves three enzymes: β-ketothiolase, acetoacetyl-CoA

Product	Yield	Strain	References
PHB	600–4,600 ng mg ⁻¹ dcw	<i>Synechocystis</i> cf. <i>salina</i> 192	[104]
	Up to 19% dcw	<i>Synechocystis</i> sp. PCC 6803	[111]
	14–30% dcw	<i>Nostoc</i> sp. PCC 7120	[99]
	5.6% dcw	<i>Synechocystis</i> sp.	[112]
	26.1% dcw	<i>Synechococcus</i> sp.	
	4.6% dcw	<i>Chlorogloeopsis fritschii</i>	[113]
	1.1% dcw	<i>Calothrix</i> sp.	
	11.4% dcw	<i>Synechocystis</i> sp.	
	63–81% dcw	<i>Synechocystis</i> sp. PCC 6803	[103]
	0.51% dcw	<i>Spirulina platensis</i>	[114]
	8.6% dcw	<i>Nostoc muscorum</i>	
	0.06 g g _{biomass} ⁻¹ d ⁻¹	<i>Anabaena</i> sp.	[115]
	13–16.4% dcw	<i>Synechocystis</i> sp. PCC 6714	[102]
	5.04% dcw	<i>Synechocystis</i> sp.	[116]
	8% dcw	<i>Synechocystis</i> PCC 6803	[117]
	17.31% dcw	<i>Oscillatoria salina</i>	[118]
	15.22% dcw	<i>Anabaena cylindrica</i>	
	14.95% dcw	<i>Nostoc linckia</i>	
	26–35% dcw	<i>Synechocystis</i> sp. PCC 6803	[101]
	28.2–39% dcw	<i>Arthrospira platensis</i> NLHT3	[119]
	Up to 85% dcw	<i>Aulosira fertilissima</i>	[105]
	34.9% dcw	<i>Synechococcus elongatus</i> PCC 7942	[109]
	3–11% dcw	<i>Synechocystis</i> cf. <i>salina</i> Wislouch	[108]
	Up to 16% dcw	<i>Synechocystis</i> sp. PCC 6714	[107]
	Up to 41.72% dcw	<i>Synechocystis</i> sp. PCC 6803	[110]
	Up to 3.1% dcw	<i>Synechocystis</i> sp. PCC 6803	[106]

dcw: dry cell weight; d: day

Table 2.
List of cyanobacteria used in polyhydroxybutyrate (PHB) production.

dehydrogenase, and PHA synthase. Acetyl-CoA is converted to acetoacetyl-CoA by β -ketothiolase, which is then converted to R-3-hydroxybutyryl-CoA by acetoacetyl-CoA dehydrogenase. PHA synthase then polymerizes this to PHB [92]. However, the rate of PHB production in cyanobacteria, especially in photoautotrophic conditions, is much lower compared to other host species [98, 99]. Recent studies show that cyanobacteria can accumulate PHB up to 85% of their dry cell weight (dcw) in nutrient-deficient and supplemented conditions [100]. For example, growing cyanobacteria in nitrogen and phosphorus-depleted conditions shows enhanced PHB accumulation [101–104]. In addition to nutrient-deprived conditions, growth media supplemented with acetate, citrate, glucose, and valerate enhance PHB production in cyanobacteria by up to 85% dcw [103, 105]. Moreover, genetic and metabolic modifications, along with carbon flux rewiring, have shown increased PHB production

in various cyanobacterial species [99, 103, 106–110]. Although bio-based plastics are more cost-effective and environmentally friendly, their production is far below expected demand. Implementing the aforementioned methods, as well as optimizing environmental and growth conditions, could alleviate the low-yield issue in cyanobacteria.

2.3 Biofertilizer

Agriculture is the only option for feeding the world's growing population. However, modern agriculture heavily relies on chemical fertilizers and pesticides to increase the quantity of agricultural products, which are not eco-friendly as they have negative impacts on the environment and human health [120, 121]. Furthermore, the production cost is high due to the rising prices of chemical fertilizers and pesticides, which have an indirect effect on the global economy. As a result, it is preferable to seek alternatives to this chemical-based farming method. Many agricultural farms are now practicing sustainable farming to ensure a safe environment and healthy food. Sustainable farming is a new method of cultivating crops and producing food that eliminates the need for chemical fertilizers. It has numerous advantages, including producing healthy and safe food at a lower cost, preserving the natural microbial population in the soil, and being eco-friendly without endangering the environment [122]. Cyanobacteria have been considered a potential candidate for sustainable farming because of their ability to increase soil fertility, protect crop plants from various pathogens, prevent heavy metals from entering the food chain, promote plant growth hormone production, and facilitate mineral solubilization and uptake from soils to plants [123–127] (**Table 3**) (**Figure 2**).

Cyanobacterial species such as *Nostoc*, *Calothrix*, *Oscillatoria* sp., *Rivularia*, *Westiella*, *Fischerella*, *Gloeotirchia*, *Scytonema*, *Tolypothrix*, *Anabaena*, and *Aulosira* have specialized cells called heterocysts that contain nitrogenase, an enzyme involved in nitrogen fixation [141–143]. However, nitrogenase is oxygen sensitive and becomes inactive when exposed to it [144]. As a result, cyanobacteria have evolved various mechanisms to avoid inactivation, including creating an oxygen-deprived environment, performing nitrogen fixation at specific times and periods, developing oxygen-tolerant nitrogenase, and overexpressing nitrogenase in the cells [138, 145–149]. Nitrogen fixation rates vary among cyanobacterial species and are also affected by environmental conditions. The average rate of nitrogen fixation by cyanobacteria is 0–1.0 nmol h⁻¹ L⁻¹ [135]. Cyanobacterial nitrogen fixation in agricultural fields, particularly in rice fields, is very high. In addition to their nitrogen-fixing ability, some cyanobacterial species, such as *Anabaena*, *Phormidium*, *Calothrix*, *Trichormus*, *Chlorogloeopsis*, *Spirulina*, *Cylindrospermum*, *Glactotheca*, *Nostoc*, *Plectonema*, and *Synechocystis* secrete a variety of chemicals into their surroundings, including abscisic acids, auxin, amino acids, and cytokinins, which promote plant growth and development [9, 143] (**Table 4**). These chemicals play numerous roles in plant growth and development [150–153]. Using cyanobacteria in agriculture has numerous benefits, including enriching the soil with various essential components that promote plant growth and development while also protecting the soil from erosion. Cyanobacteria not only promote plant growth, but they also protect them from pathogens. They produce a wide range of bioactive compounds that have antibacterial, antifungal, and antiviral properties [154, 155]. For example, cyanobacterial crude extracts have been shown in numerous studies to

Nitrogen fixation rate	Strain	References
0.67 nmol N $0.1 \text{ g}^{-1} \text{ dcw h}^{-1}$	<i>Nostoc commune</i>	[128]
0.08 $\mu\text{mol N g}^{-1} \text{ Chl s}^{-1}$	<i>Nostoc punctiforme</i>	[129]
0.25 $\mu\text{mol N g}^{-1} \text{ Chl s}^{-1}$	G23 (<i>Nostoc</i> NIES 211)	
0.26 $\mu\text{mol N g}^{-1} \text{ Chl s}^{-1}$	G42 (<i>Anabaena cylindrica</i>)	
0.027–2.85 nmol N $\text{min}^{-1} \text{ g}^{-1} \text{ FW}$	<i>Nostoc punctiforme</i>	[130]
8.9–83.9 mg N $\text{L}^{-1} \text{ d}^{-1}$	<i>Nostoc</i> sp. PCC 7120	[131]
31.4 nmol N $\mu\text{g}^{-1} \text{ Chl d}^{-1}$	<i>Oscillatoria</i> sp.	[132]
0.12–0.17 fmol N heterocyst h^{-1}	<i>Calothrix rhizosoleniae</i> and <i>Richelia intracellularis</i>	[133]
10.04	<i>Schizothrix</i>	[134]
16.6 nmol N $\text{mg}^{-1} \text{ chl h}^{-1}$	<i>Rivularia</i>	
0–0.85 nmol N $\text{L}^{-1} \text{ h}^{-1}$	<i>Nodularia</i> and <i>Dolichospermum</i>	
68–301.5 nmol N $\text{mg}^{-1} \text{ Chl h}^{-1}$	<i>Thermoleptolyngbya</i> sp. O-77	[136]
0.89 nmol N $\text{mg}^{-1} \text{ dcw h}^{-1}$	<i>Trichormus variabilis</i> K-31	[137]
0.70 nmol N $\text{mg}^{-1} \text{ dcw h}^{-1}$	<i>Nostoc</i> sp. J-14	
0.55 nmol N $\text{mg}^{-1} \text{ dcw h}^{-1}$	<i>Tolypothrix tenuis</i> J-1	
0.34 nmol N $\text{mg}^{-1} \text{ dcw h}^{-1}$	<i>Cylindrospermum badius</i> J-8	
22.0 $\mu\text{mol mg}^{-1} \text{ Chl h}^{-1}$	<i>Fischerella</i> sp.	
1.36 nmol N $\mu\text{g}^{-1} \text{ Chl h}^{-1}$	<i>Hapalosiphon</i>	[139]
0.23 nmol N $\mu\text{g}^{-1} \text{ Chl h}^{-1}$	<i>Chlorogloeopsis</i> -354	
0.64 nmol N $\mu\text{g}^{-1} \text{ Chl h}^{-1}$	<i>Westiellopsis</i>	
50.34 U $\text{mg}^{-1} \text{ dcw}$	<i>Anabaena</i>	[140]
75.33 U $\text{mg}^{-1} \text{ dcw}$	<i>Desmonostoc muscorum</i>	

FW: fresh weight; dcw: dry cell weight; Chl: chlorophyll

Table 3.
List of cyanobacteria performing nitrogen fixation.

protect plants from pathogens [156–159]. Furthermore, cyanobacteria can improve aeration and fertility of various types of soils, including salty soils [160–163]. Although cyanobacteria offer numerous benefits for sustainable agriculture, they should be used with caution as they produce cyanotoxins, which are harmful to both aquatic animals and humans [164, 165].

2.4 Bioremediation

Cyanobacteria are extremely beneficial for sustainable agriculture because they provide various nutrients, growth factors, and pathogen protection to plants [125]. They not only protect and promote plant growth and development, but they are also excellent candidates for bioremediation [171–173]. They can scavenge many toxic substances from the environment, including heavy metals, toxic industrial effluents, and other hazardous substances [174–177] (Table 5) (Figure 3). Cyanobacteria perform bioremediation through a variety of processes, including biosorption, biotransformation, and bioaccumulation [173]. Biosorption is a process where

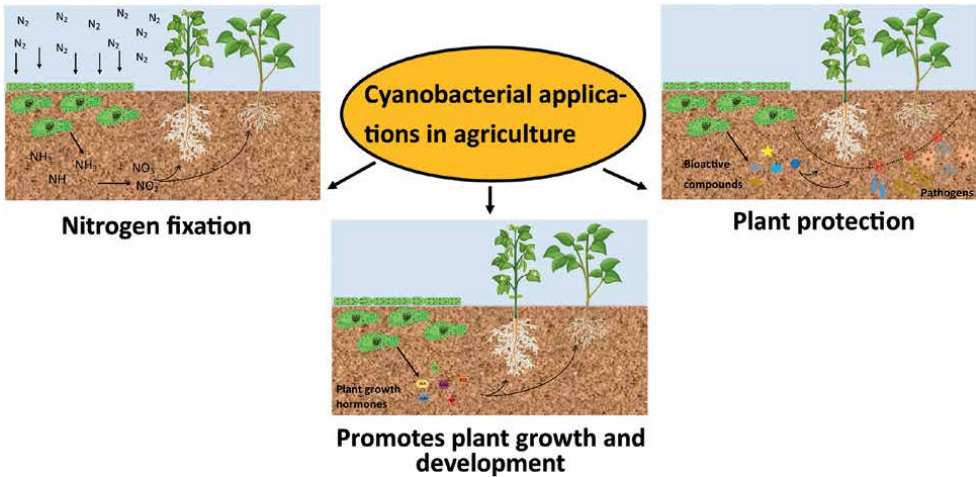


Figure 2. Cyanobacteria applications in sustainable agriculture. Cyanobacterial applications include fixing atmospheric nitrogen, enhancing soil fertility and aeration, providing plant protection, and promoting plant growth and development.

Product	Yield	Strain	References
Auxin	5.5 $\mu\text{g g}^{-1}$ FW	<i>Planktothricoides raciborskii</i>	[166]
	0.9 $\mu\text{g mL}^{-1}$	<i>Leptolyngbya</i> SM-13	[167]
	2.5 $\mu\text{g mL}^{-1}$	<i>Chroococcidiopsis</i> SM-04	
	4.0 $\mu\text{g mL}^{-1}$	<i>Phormidium</i> SM-14	
	3.6 $\mu\text{g mL}^{-1}$	<i>Synechocystis</i> SM-10	
	9.4 pmol g^{-1} FW	<i>Nostoc microscopium</i>	[168]
	192.9 pmol g^{-1} FW	<i>Chroococcus minutus</i>	
Cytokinin	250.1 pmol g^{-1} FW	<i>Phormidium animale</i>	
	0.0063 $\mu\text{g mL}^{-1}$	<i>Nostoc</i>	[169]
	0.0059 $\mu\text{g mL}^{-1}$	<i>Anabaena</i>	
Absciscic acid	1.0 $\mu\text{g L}^{-1}$	<i>Synechococcus</i> sp.	
		<i>Synechococcus leopoliensis</i> , <i>Nostoc muscorum</i> , <i>Trichormus variabilis</i>	[170]

FW: fresh weight

Table 4. List of cyanobacteria produce plant hormones.

cyanobacteria passively bind heavy metals through extracellular polysaccharides on their cell walls. Cyanobacterial species from the genera *Synechococcus*, *Nostoc*, *Spirulina*, *Oscillatoria*, *Microcystis*, and *Phormidium* have been found to be effective at accumulating heavy metals from aqueous solutions, including Ni, Cu, Pb, Cd, and Zn [178–184]. The rate of biosorption in cyanobacteria ranges between 2 and 515 mg g^{-1} dcw [185].

Rate	Name of chemical/metals	Strain	References
18.19 mg g ⁻¹ biomass	Nickel (Ni ²⁺)	<i>Chroococcidiopsis</i> sp.	[203]
13.40 mg g ⁻¹ biomass		<i>Arthrospira platensis</i>	[204]
38.0–63.80%		<i>Nostoc</i> sp.	[205]
98.03 mg g ⁻¹ biomass	Zinc (Zn ²⁺)	<i>Fischerella ambigua</i> ISC67	[206]
89–95%		<i>Microcystis aeruginosa</i>	[184]
1.80 mg g ⁻¹ biomass		<i>Nostoc commune</i> R123	[183]
1.70 mg g ⁻¹ biomass		<i>Nostoc commune</i> AB55	
2.0 mg g ⁻¹ biomass		<i>Nostoc commune</i> NR64	
82–87%		<i>Nostoc</i> sp.	[205]
80–100%		<i>Nostoc linckia</i>	[207]
92.27–98.15%	Lead (Pb ²⁺)	<i>Nostoc</i> sp.	[205]
89–95%		<i>Microcystis aeruginosa</i>	[184]
3.90 mg g ⁻¹ biomass		<i>Nostoc commune</i> R123	[183]
2.40 mg g ⁻¹ biomass		<i>Nostoc commune</i> AB55	
1.80 mg g ⁻¹ biomass		<i>Nostoc commune</i> NR64	
86%		<i>Microcystis aeruginosa</i>	[208]
90–100%		<i>Microcystis aeruginosa</i>	[209]
95.14–96.0%	Copper (Cu ²⁺)	<i>Nostoc</i> sp.	[205]
Up to 45%		<i>Cyanothece</i> sp. CE4	[210]
65–90%		<i>Nostoc linckia</i>	[207]
0.0 mg g ⁻¹ biomass		<i>Nostoc commune</i> R123	[183]
4.10 mg g ⁻¹ biomass		<i>Nostoc commune</i> AB55	
1.20 mg g ⁻¹ biomass		<i>Nostoc commune</i> NR64	
92%	Cadmium (Cd ²⁺)	<i>Microcystis aeruginosa</i>	[208]
4.0–17.7 mg g ⁻¹ biomass		<i>Synechocystis</i> sp. PCC6803	[211]
93.4%		<i>Nostoc muscorum</i>	[212]
89.13%		<i>Trichormus variabilis</i>	[212]
69.5–98.4%		<i>Microcystis aeruginosa</i>	[213]
80–100%		<i>Microcystis aeruginosa</i>	[209]
91.0%		<i>Nostoc</i> sp.	[205]

Rate	Name of chemical/metals	Strain	References
60.7–92.4%	Aluminum (Al^{3+})	<i>Microcystis aeruginosa</i>	[213]
73–100%	Iron (Fe^{2+})	<i>Nostoc linckia</i>	[207]
84.2–91.5 mg g ⁻¹ biomass	Cerium (Ce^{3+}), Neodymium (Nd^{3+}), Terbium (Tb^{3+}), Lanthanum (La^{3+})	<i>Nostoc</i> sp. 20.02	[214]
69.5–83.4 mg g ⁻¹ biomass		<i>Synechococcus elongatus</i> UTEX 2973	
68.6–83.5 mg g ⁻¹ biomass		<i>Calothrix brevissima</i> SAG 34.79	
44.7–70.6 mg g ⁻¹ biomass		<i>Desmonostoc muscorum</i> 90.03	
47.2–67.1 mg g ⁻¹ biomass		<i>Komarekiella</i> sp. 89.12	
63%	Chromium (Cr^{6+})	<i>Limnococcus</i> sp.	[192]
96%		<i>Spirulina fusiformis</i>	
92.91%	Uranium (U^{6+})	<i>Anagnostidinema amphibium</i>	[215]
25.4–56.27 mg g ⁻¹ biomass	Cesium (Cs)	<i>Synechococcus</i> PCC 7002	[216]
62–90%	Strontium (^{90}Sr)	<i>Arthrospira platensis</i> CNMN–CB-02	[217]
15–99.7%	Neptunium (^{237}Np)		
58%	Cesium (^{137}Cs)		
3–8%	Uranium (^{233}U)		
67–98.7	Plutonium (^{239}Pu)		
77–99.4%	Americium (^{241}Am)		

Table 5.
 List of cyanobacteria used in bioremediation.

Furthermore, some cyanobacteria have been shown to be effective in removing a variety of toxic metals from wastewater and industrial effluents through bioaccumulation [186–189]. Cyanobacteria transport heavy metals from their surroundings to their cells using various passive and active transporters, as well as ion channels in the membrane [173]. The rate of bioaccumulation varies by species and metal type. For example, the bioaccumulation efficiency of *Nostoc* sp. for Cd^{2+} , Hg^{2+} , and Pb^{2+} is 29%, 34%, and 34%, respectively, whereas the efficiency rates of *Synechococcus* sp. PCC 7942 for the same metals are 34%, 50%, and 45%, respectively [190]. Similarly, *Limnococcus* sp. and *Spirulina fusiformis* have a bioaccumulation efficiency of 63% and 96% for Cr^{6+} , respectively [191, 192]. Cyanobacteria not only accumulate various toxic metals from their surroundings, but they also detoxify and convert them into less toxic forms within the cell. They biotransform toxic metals using repeated oxidation and reduction reactions.

Current agricultural methods heavily rely on pesticides and herbicides to protect crop plants from pathogens and wild flora. As a result, nearby water bodies become contaminated with pesticides and herbicides. Cyanobacteria have shown promise in degrading these chemicals from polluted water. Cyanobacteria such as *Nostoc muscorum*, *Microcystis aeruginosa*, *Anabaena*, and *Fischerella* sp. have been

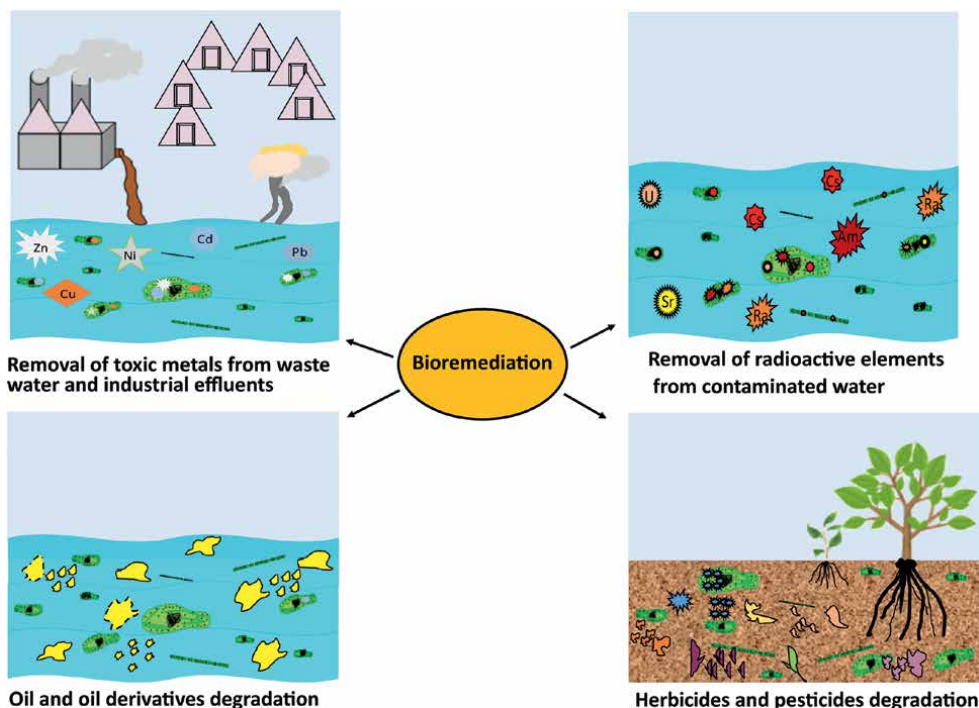


Figure 3.

Overview of cyanobacterial applications in bioremediation. Cyanobacterial applications include the removal of toxic metals from waste water and industrial effluents, the removal of radioactive elements from contaminated water, the degradation of oil and oil derivatives from water, and the degradation of herbicides and pesticides from contaminated soil.

demonstrated to successfully degrade pesticides from agricultural fields and contaminated water bodies [193–196]. Similarly, numerous studies have utilized cyanobacteria as hosts to degrade herbicides from polluted soil. Cyanobacteria, such as *Anabaena*, *Microcystis*, *Nostoc*, *Synechococcus elongatus*, and *Spirulina*, have shown promising results in breaking down herbicides from their surrounding contaminated soil [197–202]. Although they are naturally potent at removing toxic chemicals from the environment, the rate of removal is not high and can be improved through optimization. Thus, cyanobacteria could be a viable candidate for large-scale bioremediation in the near future.

3. Conclusion

Cyanobacteria have great potential for biomanufacturing, as well as for sustainable agriculture and bioremediation. Numerous studies using cyanobacteria as hosts have shown promising results in terms of bioproduct generation, toxic pollutant and metal removal from the environment, and plant growth. However, product yield, contamination, and health risks remain hurdles to large-scale applications. Genetically modified strains are capable of enhancing bioproduct formation, toxic metal removal, and nitrogen fixation. However, the use of genetically engineered strains is associated with environmental risks. As a result, the

adaptive laboratory evolution method can be used as an alternative to genetic modification for developing highly efficient strains. Thus, the use of highly efficient strains, combined with optimized downstream processing, could alleviate current challenges and transform cyanobacteria into a cost-effective biological chassis for a variety of applications.

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Structural and Functional Roles of the Inorganic Cofactors of Photosystem II

Leslie Castillo, Nidhi Patel, Nicole Seliga, Gustavo Chavez and Colin Gates

Abstract

Photosystem II (PSII) is the sole water oxidase in nature. This large, multisubunit enzyme complex relies on several critical inorganic cofactors for its operation, namely manganese, calcium, chloride, bicarbonate, and iron. In this review, we address the state of the field with regard to the roles of these cofactors in the operation and functionalization of PSII. We specifically address recent developments in our understanding of the water-oxidizing complex (WOC), the catalytic site of water oxidation, and the processes regulating the oxidation states of the catalytic manganese and the role of redox-inert calcium; the function of chloride near the WOC; and the role of the nonheme iron and its necessary bicarbonate ligand in regulating electron transfer between the reaction center and the terminal electron acceptor Q_B .

Keywords: Photosystem II, electron transport, water oxidation, chloride, bicarbonate

1. Introduction

Oxygenic photosynthesis is the fundamental biological process that sustains nearly all life on Earth by converting sunlight into chemical energy. This innovation transformed Earth's atmosphere and enabled the evolution of aerobic life [1]. Oxygenic photosynthesis is carried out by diverse organisms, including cyanobacteria, algae, and plants, whose ability to split water and power electron transport drives global oxygen and biomass production. The capacity of phototrophs to oxidize water and drive linear electron transport originates from Photosystem II (PSII), a large multisubunit protein complex embedded in the thylakoid membrane [2]. PSII catalyzes the light-driven oxidation of water at the water-oxidizing complex (WOC) and the reduction of plastoquinone at the Q_B binding site, producing molecular oxygen, protons and electrons that sustain photosynthetic electron flux [3–5]. It remains the only known enzyme capable of oxidizing water, a process initiated by photon absorption at chlorophyll pigments

and stabilized through long-lived charge separation by the reaction center, P680 [2]. Integration of PSII photochemistry with the WOC enables the system to generate both redox products, molecular oxygen and reduced plastoquinol (PQH_2), and a proton gradient across the thylakoid membrane that contributes to the proton motive force that powers ATP synthesis [3, 5]. The catalytic core of the WOC consists of an inorganic Mn_4CaO_5 cluster coordinated within the protein matrix, which accumulates oxidizing equivalents across four charge separation events to drive four-electron oxidation of two water molecules [4]. The operation of PSII is indispensable not only for energy conversion in phototrophs but also for sustaining Earth's oxygenic atmosphere. Despite phototroph phylogenetic and ecological diversity, the inorganic core and protein architecture of PSII remain conserved.

Structurally, PSII is a large membrane protein complex with a highly conserved core of subunits surrounded by less essential and sometimes transient proteins (**Figure 1**). The dimeric holoenzyme contains over 30 protein subunits together with a network of pigments, lipids, and inorganic cofactors. The reaction center is formed by the D1 (PsbA) and D2 (PsbD) proteins, which coordinate the primary electron-transfer cofactors, including the special pair of chlorophylls (P680), pheophytins, plastoquinones at the Q_A and Q_B sites, and the nonheme iron (NHI) [6]. The core antenna protein subunits CP43 and CP47 (PsbC and PsbB) coordinate over 30 chlorophyll molecules and carotenoids that funnel excitation energy into the reaction center [7]. Several low-molecular-mass subunits and extrinsic luminal proteins further stabilize the complex and regulate water oxidation [8]. Among these, PsbO is conserved as the manganese stabilizing protein, whereas other extrinsic components

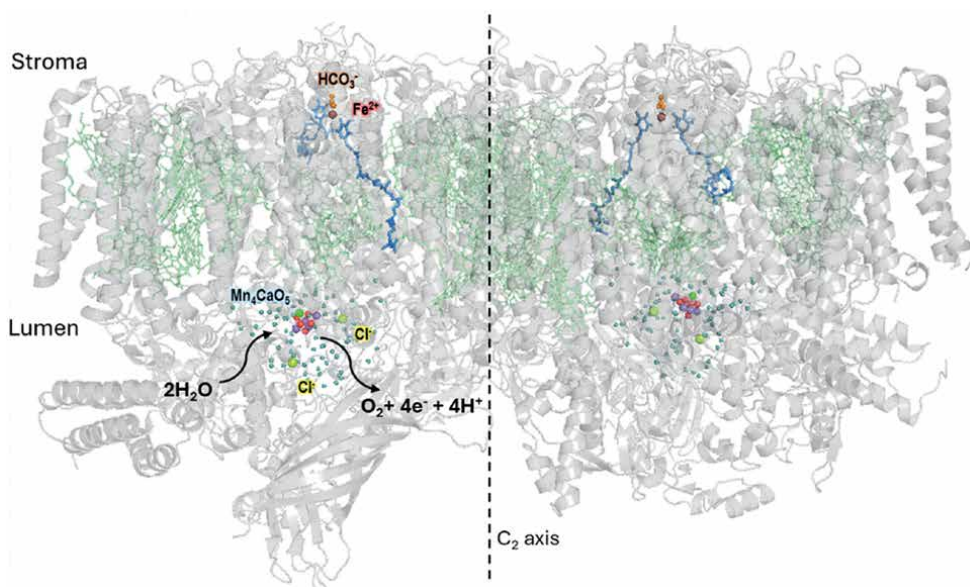


Figure 1.

PSII structure highlighting known positions of relevant cofactors, including the WOC with CA (green), MN1-4 (purple), and bridging oxo ligands (red); two donor-side chloride ions (yellow), the NHI (ruby), and its bicarbonate (orange). Chlorophylls are shown in light green, plastoquinone acceptors in dark blue, and water molecules in cyan. Structure from PDB 78no.

vary by lineage [9]. High-resolution structures of cyanobacterial PSII from *Thermosynechococcus elongatus* and *Synechocystis* sp. PCC 6803 have revealed the spatial organization of protein subunits, pigments, and the Mn_4CaO_5 cluster [10–12]. Structures resolved to near-atomic detail by X-ray crystallography and cryo-electron microscopy provide the foundation for understanding how PSII integrates light harvesting, electron transfer, proton translocation, and catalysis, illuminating how protein architecture supports the chemistry of water oxidation [13–15].

While the protein subunits of PSII provide the structural framework, its catalytic activity depends on a precisely tuned network of inorganic cofactors (**Figure 1**). On the donor side, the Mn_4CaO_5 WOC catalyzes the four-electron oxidation of water to O_2 through the Kok S-state cycle, advancing through five metastable oxidation states (S-states, $\text{S}_0 \rightarrow \text{S}_1 \rightarrow \text{S}_2 \rightarrow \text{S}_3 \rightarrow [\text{S}_4]$) that sequentially accumulate oxidizing equivalents on the redox-active manganese until O–O bond formation and oxygen release occur [5, 16]. Calcium (Ca^{2+}) plays a critical role in stabilizing the cluster geometry and facilitating substrate water binding, while a hydrogen-bonded network of waters and residues coordinates proton removal [17]. Chloride (Cl^-) ions further contribute by maintaining the integrity of this network and enabling efficient proton egress [18]. On the acceptor side, bicarbonate (HCO_3^-) bound near the NHI modulates quinone redox potential, stabilizes forward electron transfer, and minimizes charge recombination pathways [19–21]. Collectively, these cofactors enable PSII to accomplish efficient water oxidation, maintain redox poise, and minimize photodamage.

1.1 Significance

Despite decades of study, the precise mechanistic contributions of these cofactors remain under active investigation. Herein we examine the roles of five inorganic cofactors in PSII: manganese, calcium, chloride, bicarbonate and the NHI (**Table 1**). We emphasize their operational roles in catalysis, electron and proton transfer, and structural stabilization rather than in photoassembly, which has been covered in detail in previous reviews [17, 22, 23]. As presently stands, major questions remain regarding the functions of all of these inorganic cofactors. Therefore, their roles must be elucidated to provide a strong foundation for future research efforts to improve the efficiency of PSII in natural systems, a critical objective for improving crop and biofuel productivity, or develop efficient biomimetic water-oxidizing systems,

Cofactor	Thylakoid position	Role
Manganese	Lumen (WOC)	Redox cycling, water oxidation
Calcium	Lumen (WOC)	Tuning of manganese operation
Chloride	Lumen (near WOC)	Proton removal from the WOC
Bicarbonate	Both sides (NHI)	Mediates protonation, electron-transfer Q_A – Q_B , and WOC photoassembly
Iron	Stroma (Q_A – Q_B)	Mediates electron-transfer Q_A – Q_B

Table 1.
List of inorganic cofactors of PSII, their locations, and roles.

a longstanding goal in renewable energy technology. In the following sections, we discuss each cofactor individually, emphasizing structure of the ligand environment, mechanistic role in PSII, and remaining questions on operation. By integrating structural and mechanistic perspectives, this chapter aims to provide a clear framework for understanding what is known and what is not presently known about how these cofactors regulate water oxidation and maintain PSII function.

2. Manganese

2.1 Structural location

The WOC of PSII comprises the Mn_4CaO_5 cluster, an inorganic cofactor that drives light-driven water oxidation (**Figure 2**). Embedded in a well-defined pocket on the luminal side of the complex, the cluster adopts a cubane-like geometry wherein three manganese ions (Mn1-Mn3) and a single calcium ion form a heterocubane core, while a fourth “dangler” manganese (Mn4) is linked to the others through μ -oxo bridges [10]. Four terminal waters (W1-W4) coordinate to Mn4 and Ca^{2+} and are essential for O_2 evolution. The cluster is ligated primarily by D1 subunit residues including D1-D170, D1-E189, D1-E333, D1-D342, D1-A344, D1-H337, and D1-H332, along with CP43-E341 (**Figure 2A**). Several of these carboxylates are bridging ligands to adjacent Mn and Ca^{2+} , while D1-H332 and D1-E189 ligate Mn1 in a monodentate manner [24]. Notably, D1-D170 provides a high-affinity site for the first Mn^{2+} and oxidation during the photoassembly of the WOC [25]. These μ -oxo bridges, terminal ligands, and carboxylate coordination create a stable yet redox-flexible framework that stabilizes high-valent manganese while allowing storage of multiple oxidizing equivalents.

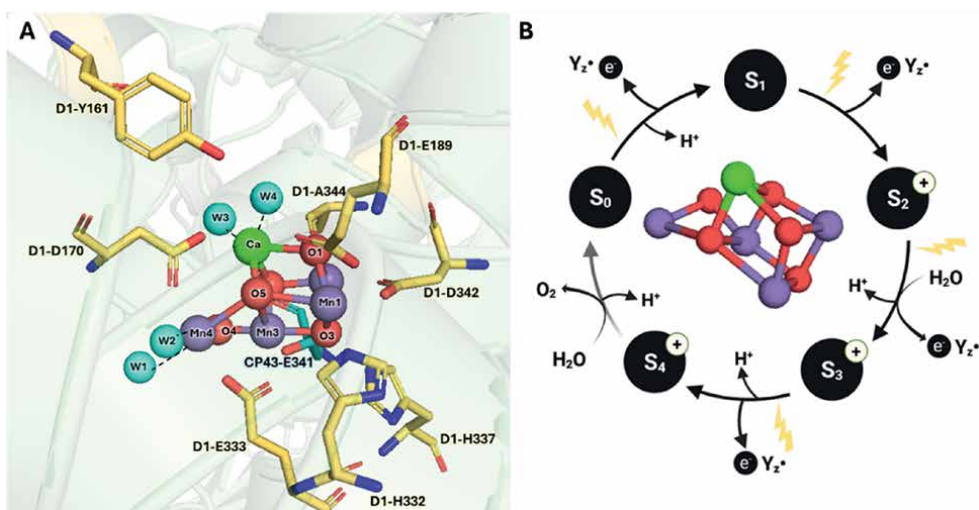


Figure 2.

(A) The WOC of PSII, showing the Mn_4CaO_5 cluster with conventional numerical assignments of atoms, the four conserved water ligands to Mn4 and Ca, and the ligands of the first coordination sphere, as well as tyrosine-z for directional reference. (B) The WOC cycle of S-state transitions showing oxidation, deprotonation, and water uptake events.

High-resolution crystallography revealed the geometry of the cluster as a Mn_3CaO_4 pseudocubane linked to a terminal manganese ion [2, 10]. These breakthrough structures established the foundation for current models but have well-known limitations: The manganese ions are prone to reduction under synchrotron Xrays, the exact positions of μ -oxo and water ligands remain difficult to resolve because oxygen scatters weakly relative to the surrounding metals, and the protonation states of the oxygens within the WOC remain unknown, being unobservable with current approaches [15, 26, 27]. Complementary spectroscopic methods have provided key constraints: Extended Xray absorption fine structure demonstrated metal–metal and metal–ligand distances, while electron paramagnetic resonance (EPR) and ENDOR spectroscopy assigned manganese spin states across the Kok cycle [28, 29]. Computational studies using DFT and QM/MM integrate experimental results to generate several models that do not completely align [30, 31].

2.2 Redox cycling and catalytic function

The functional role of Mn in PSII is defined by its ability to undergo sequential one-electron redox transitions. Within the Mn_4CaO_5 cluster, the Mn ions cycle primarily between the +3 and +4 oxidation states, allowing stepwise accumulation of four oxidizing equivalents required for water splitting [28]. Each charge separation event generates the strong oxidant P680^+ , which is quickly reduced by the redox-active tyrosine TyrZ (D1-Y161) [32]. Upon donating an electron, TyrZ forms a neutral radical ($\text{TyrZ}\cdot$) which transfers the oxidizing equivalent to the manganese cluster through the hydrogen-bonded D1-H190 network [16]. This rapid sequence ensures the oxidizing power generated by light absorption is stabilized and stored at the catalytic site. Each manganese oxidation step is coupled to proton release from μ -oxo or water ligands, preventing excessive charge accumulation and maintaining the energetic balance [33].

2.3 Manganese in the S-state transitions

The four manganese ions in the Mn_4CaO_5 cluster sequentially accumulate the oxidizing equivalents required for water splitting. Progression through S-state transitions drives stepwise electron removal, proton release, and water substrate activation, ultimately enabling O=O bond formation [34, 35]. Although it is known that the S-states advance through successive light driven charge separation events, the exact electronic configuration of the metal ions at each step remains under investigation. The high covalency and charge delocalization of the cluster complicates oxidation state assignments, and while spectroscopic, crystallographic, and computational studies have provided insights, they also highlight areas where interpretations remain unresolved [13, 28]. Early spectroscopic work demonstrated that each S-state transition is linked to electron removal and proton release, but subsequent studies indicated that proton release is not uniform across the cycle, producing a characteristic 1:0:1:2 pattern of proton stoichiometry (starting from the $\text{S}_0 \rightarrow \text{S}_1$ transition) [36, 37].

The $\text{S}_0 \rightarrow \text{S}_1$ transition involves the oxidation of the most reduced state of the cluster to the dark stable S_1 state. It is associated with the loss of one electron and one proton, preparing the cofactor for further charge accumulation steps in the cycle. Spectroscopic evidence including EPR and ENDOR studies suggests that the S_1 state

is diamagnetic and represents the resting state under physiological conditions [13, 32]. While the exact oxidation configuration of the manganese ions in S_0 and S_1 remains debated across different experimental and computational models [28], there is a broad agreement that this transition establishes the baseline state from which the water oxidation cycle proceeds.

The $S_1 \rightarrow S_2$ transition is initiated by oxidation of the water oxidizing complex via $Y_Z \cdot$, resulting in the removal of an additional electron from the cluster without an associated proton release [34, 38]. This is distinct within the Kok cycle because it is the only step when net charge accumulates on the cofactor. EPR spectroscopy has characterized the S_2 state, revealing two interconvertible spin isomers: a multiline signal at $g \approx 2$ and a broad feature at $g \approx 4.1$ [39, 40]. These signals are commonly associated with “open” and “closed” cubane conformations of the Mn_4CaO_5 cluster [41]. X-ray free electron laser (XFEL) studies have provided structural evidence consistent with both configurations, leaving unresolved which conformation predominates under physiological conditions [42, 43]. The existence of multiple electronic and structural isomers emphasizes the flexibility of the S_2 state, which is proposed to enable water insertion and proton release during the successive transition [44].

The transition from $S_2 \rightarrow S_3$ is widely recognized as a key step where the water oxidizing complex undergoes oxidation and structural rearrangement. In addition to electron transfer, this transition is associated with proton release, reflecting changes in the hydrogen-bond network surrounding the Mn_4CaO_5 cluster [34]. Structural studies using femtosecond X-ray crystallography have reported an additional oxygen ligand (O6), in the S_3 state [42, 43, 45, 46]. Its positioning near O5 led to proposals that O6 could function as a substrate oxygen. Early structural models suggested a short O5–O6 distance, posited as O–O bond formation [45]. However, high-resolution studies have reviewed this distance, indicating that O–O formation has not occurred at this stage [43, 47]. Various models describe O6 as a ligand bound to manganese, as a water molecule from the Ca^{2+} site, or as a preloaded substrate for the next cycle [4, 48]. While the presence and function of O6 is uncertain, the $S_2 \rightarrow S_3$ transition is recognized as a step for structural and electronic reorganization that prepares the cluster for oxygen evolution.

The final $S_3 \rightarrow [S_4] \rightarrow S_0$ transition is when O=O bond formation and the release of two protons and O_2 occur, with a reset of the Mn_4CaO_5 cluster. The S_4 state itself is a highly transient intermediate, detected only indirectly through time-resolved spectroscopy and kinetic studies, which has made its electronic characterization difficult [35, 43]. Time-resolved X-ray spectroscopy and fluorescence studies have suggested that after Y_Z oxidation, an intermediate state persists for hundreds of microseconds before O_2 release, a period associated with a proton release [38, 49]. The exact electronic configuration of this intermediate remains debated: Some mechanistic models propose a Mn-oxyl species that undergoes oxo–oxyl coupling, while other groups propose a formation of a high-valent Mn-oxo intermediate that undergoes nucleophilic attack by bound hydroxide or water [50]. In both cases, structural studies suggest O5 as one substrate oxygen, while the second may correspond to a newly inserted ligand such as O6 or a calcium-bound water seen in XFEL structures [14, 42, 43]. While the exact pathway remains unknown, it is agreed that that proton releases occur both before and after O–O bond formation and that cluster reorganization following O_2 release restores the S_0 state.

2.4 Coupling of proton and electron transfer

Manganese oxidation within the Mn_4CaO_5 cluster modulates the proton affinity of μ -oxo bridges and terminal waters, shifting the local pKa and coordinating proton release coupled to electron removal. This coupling gives rise to the characteristic 1:0:1:2 proton release pattern observed in the Kok cycle, preventing excessive charge buildup and maintaining redox potentials accessible to $\text{Y}_z\cdot/\text{Y}_z$ [5, 34]. Proton transfer is gated by Y_z oxidation and directed through hydrogen-bond networks such as the Cl^- channel, ensuring each electron transfer to P680^+ proceeds in favorable electrochemical conditions and maintains synchronicity of charge accumulation and substrate activation [28, 36]. Studies from site-directed mutagenesis, including D1–D61 and CP43–R357 variants, revealed that disruption of these networks leads to slowed S-state transitions and altered proton release, connecting residues to catalytic gating [51, 52]. Metal substitution studies further emphasized the functional distinctiveness of manganese: Substitution with cobalt disturbs charge accumulation and delays the Kok cycle before O_2 evolution, underlining the requirement for manganese's redox flexibility to support multielectron chemistry [53]. PSII has evolved a finely tuned network in which manganese oxidation, proton transfer, and Y_z -mediated electron flow are coordinated to accomplish efficient water splitting.

3. Calcium

3.1 Structural description

Within the Mn_4CaO_5 cluster of PSII, calcium occupies the corner of the WOC distorted cubane opposite Mn3 [10, 26]. The pseudocubane is not static; the cluster can exist in either an “open” or “closed” configuration in at least some S-states [54]. In the open cubane, Mn4 is connected to the core by O4 and O5, while in the closed cubane, Mn4 solely ligates O4. The open form is believed to facilitate substrate water access and O–O bond formation by maintaining Mn4 in a reactive position [28]. Calcium stabilizes the open cubane geometry through its specific coordination pattern, enforcing the arrangement for efficient catalysis. Calcium is oxo-bridged to all 4 Mn ions via di- μ -oxo bridges with Mn1 via O1 and O5, Mn2 via O1 and O2, and Mn3 via O2 and O5; and a mono- μ -oxo bridge with Mn4 via O5. Calcium also coordinates waters 3 and 4.

3.2 Function of calcium

Calcium is essential for O–O bond formation because it stabilizes high-valent Mn intermediates and provides a ligated water molecule (either W3 or W4) to potentially couple with a Mn-oxo species [55]. Calcium maintains Mn–Mn distances within a catalytically suitable range and tunes the electronic environment to facilitate proton-coupled electron transfer [56]. By sharing μ -oxo ligands with Mn ions, calcium indirectly tunes their redox potentials by lowering the energetic cost of reaching high-valent states necessary for O–O bond formation and facilitating the $\text{S}_2 \rightarrow \text{S}_3$ transition [57], as observed via FTIR studies [58]. Calcium's electrostatic field is further proposed to stabilize reactive Mn-oxo intermediates by polarizing μ -oxo ligands and enabling delocalization of charge, which stabilizes Mn(IV) intermediates in $\text{S}_2 \rightarrow \text{S}_3 \rightarrow \text{S}_4$ transition states and prevents destructive radical accumulation [32].

3.3 Atomic substitution

Substitution studies demonstrate the unique role of calcium in the structure and reactivity of the WOC. Sr^{2+} is the only ion able to replace calcium while supporting oxygen evolution [59]. Crystallographic studies demonstrate that Sr^{2+} occupies the calcium WOC site, preserving the geometry of the cluster despite its larger ionic radius [60]. Sr^{2+} substitution results in slower O–O bond formation, reduced quantum efficiency, and altered S-state lifetimes [61]. Sr^{2+} influences the WOC toward a closed cubane geometry [62]. All other divalent cations cannot replicate these functions. Cd^{2+} can be incorporated but blocks advancement through the S-states beyond the S_2 -state and distorts Mn–Mn distances [63], while no other substituent allows WOC turnover or S-state advance. Disruption of the calcium site interferes with the linkage of substrate delivery at W3/W4 to the timing of proton and electron-transfer events [58].

3.4 Remaining questions

Several aspects of calcium's function in the water oxidizing complex remain unknown despite decades of study. One outstanding question is whether calcium, as a Lewis acid, can redistribute electron density within the cluster. Calcium is proposed to displace electron density across the Mn cluster, modulating reactivity [64]. Biochemical studies indicate that incorporation of calcium guides manganese binding and oxidation in photoassembly. Omission or substitution of calcium stalls this process at intermediate states [65]. During PSII repair, partial turnover of calcium has been observed, consistent with its high stoichiometric requirement in photoassembly [66]. In addition to the catalytic site, a secondary calcium-binding site has been identified in the extrinsic PsbO subunit, which does not participate in O–O bond formation [67]. Removal of calcium from this peripheral calcium-binding pocket weakens PsbO binding, which indirectly destabilizes the WOC and reduces oxygen evolution efficiency without affecting the primary catalytic mechanisms. Mechanistically, this site may help maintain the structural integrity of loop regions that shield the WOC and regulate substrate water accessibility. It may also act as a local reservoir for calcium during photoassembly and repair cycles.

4. Chloride

Beyond metal cofactors, small anions play critical roles in PSII function. Among these, chloride has received attention due to its essential role in supporting water oxidation and stabilizing the water oxidizing complex during both catalysis and assembly. The following section summarizes the current understanding of chloride's binding sites, mechanistic roles, and impact on PSII efficiency.

4.1 Location and binding sites

Structural, spectroscopic, and computational approaches have established that PSII contains two conserved chloride-binding sites per monomer, located on the luminal side near the Mn_4CaO_5 cluster. Early studies showed that Cl^- depletion inhibits S-state transitions and revealed a strict Cl^- requirement for O_2 evolution. The

precise locations of these ions near the WOC were determined by X-ray crystallography and reinforced by cryo-EM [10, 11]. The two sites, known as Cl1 and Cl2, are located on opposite sides of the Mn_4CaO_5 cluster, approximately at $>7 \text{ \AA}$ from the nearest manganese ion and $\sim 11 \text{ \AA}$ from the calcium ion, within hydrogen-bond networks of key residues but outside the first coordination sphere (**Figure 3**) [68, 69]. Cl1 is located at the entrance of the broad (Cl1) water channel leading to the lumen and is surrounded by the side chains of D1-N181, D2-K317, and nearby D1-D61 [70]. Cl2 lies in a small pocket adjacent to the WOC near the narrow O4 channel, coordinated by the backbone of D1-N338 near the hydrogen-bond network [10]. Both sites are adjacent to ordered water networks that connect the catalytic cluster to the lumen [71]. While occupancy can vary depending on sample preparation, the binding sites are consistently observed across cyanobacterial and higher plant PSII structures [10, 11].

4.2 Functional roles

Cl^- is essential for sustaining efficient PSII turnover, particularly by facilitating proton removal from the Mn_4CaO_5 cluster to the lumen during the $\text{S}_2 \rightarrow \text{S}_3$ and $\text{S}_3 \rightarrow \text{S}_0$ transitions [72–74]. Cl^- depletion slows these transitions, leading to impaired O_2 evolution and accumulation of oxidized intermediates. Cl^- also helps to maintain the

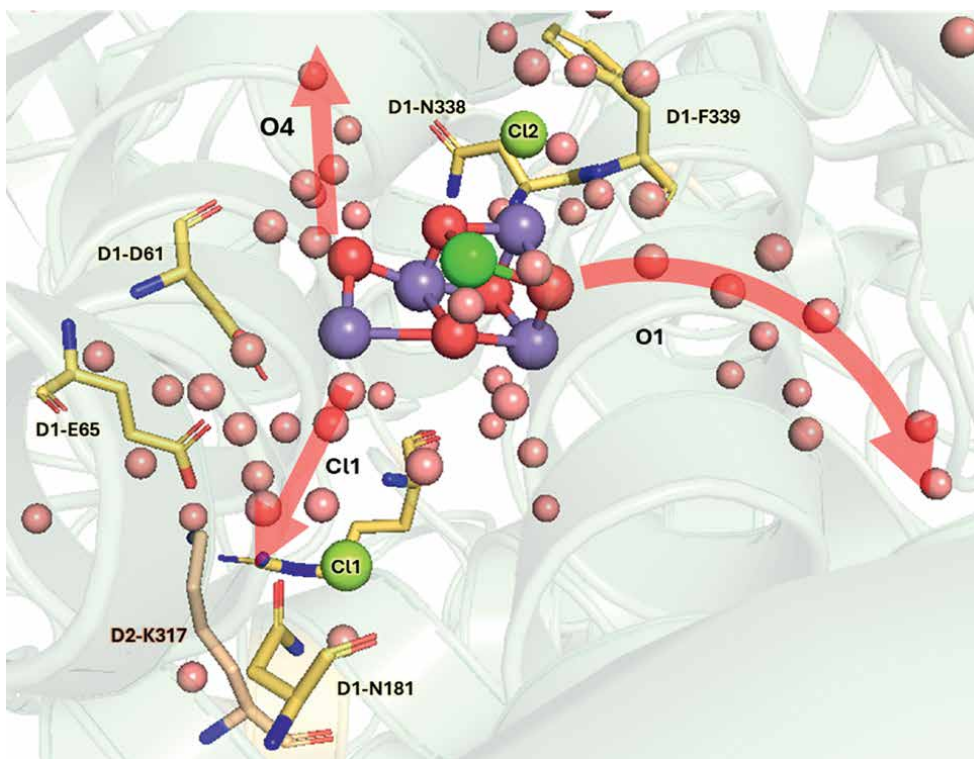


Figure 3.
 Close-up view of the canonical chloride-binding sites in PSII, highlighting both Cl1 and Cl2 positions (yellow). Surrounding channels (red) and residues from D1 (yellow) and D2 (beige) that interact with each site are shown, with hydrogen-bonding waters (salmon) displayed as spheres.

proper electrostatic environment near the catalytic site. The Cl1 site modulates the protonation states of nearby residues such as D1–D61 and stabilizes nearby hydrogen-bond networks [18]. Occupancy of this site prevents the formation of an inhibitory salt bridge between D1–D61 and D2-K317, which would block the proton egress pathway channel and halt catalysis [69, 75, 76]. Some studies further suggest that Cl⁻ contributes to stabilizing the Mn₄CaO₅ cluster and its surrounding environment, though the exact mechanism remains debated [69, 77, 78]. Evidence from halide substitution studies suggests that chloride's role is not purely structural but chemically specific and optimized for PSII, as anion substitution leads to impaired proton removal and decreases photosynthetic efficiency [73, 79]. Through these electrostatic and structural contribution effects, Cl⁻ acts as a gatekeeper of proton transfer.

4.3 Mechanistic evidence

Several studies have established a relationship between Cl⁻ binding, generally at the Cl1 site, and efficient PSII catalysis. Early chloride depletion and reconstitution experiments showed that removing Cl⁻ from isolated PSII membranes significantly reduces O₂ evolving activity and delays the WOC cycle at the S₂→S₃ and S₃→S₀ transitions where proton release occurs [80]. Readdition of chloride restores both S-state advancement and O₂ evolution, indicating that the effect is reversible and reflects a specific requirement for chloride rather than irreversible damage. Spectroscopic studies, including EPR and FTIR, reveal characteristic modifications in the S₂ multiline signal and shifts in the hydrogen-bonding network under Cl⁻ depletion [78, 81]. In addition, time-resolved fluorescence and flash-induced O₂ evolution have shown delayed proton release kinetics and increased charge recombination in the absence of Cl⁻ [68, 80].

Additional mechanistic insights have come from halide and anion substitution studies, which investigated whether Cl⁻ is chemically specific or simply structural. Bromide is the most studied replacement because of its similar size and charge, with crystal structures confirming its occupancy at the Cl1 site [69, 70, 73, 82]. Despite this, bromide slows the S₂→S₃ and S₃→S₀ transitions, delays proton release to the lumen, reduces and slows the formation of the thylakoid ΔpH, and increases charge recombination at moderate-to-high light intensities [82–84]. These effects demonstrate that Cl⁻ optimizes proton-coupled electron-transfer kinetics and minimizes recombination [84, 85]. Iodide can partially rescue O₂ evolution but slows proton release and promotes competing side reactions, including sacrificial electron donation and Y_Z modification, which disrupts electron flow [68, 83].

Mutagenesis of residues surrounding the Cl1 binding site has provided critical insight into chloride's functional role. Mutants of D2-K317 disrupt the electrostatic environment near the Cl1 channel and reduce chloride affinity, yet O₂ evolution remains partially supported [76, 86]. This lysine can form a salt bridge with D1–D61, which would block proton egress, when chloride is absent [18, 76]. In contrast, mutations at D1-N181, another residue near Cl1, do not eliminate the binding of Cl⁻ but lead to significantly reduced steady-state O₂ evolution and slower O–O bond formation despite normal S-state cycling [87]. These results imply that D1-N181 tunes the geometry of water ligands and the hydrogen-bond network rather than acting as a gate in proton transfer. Altogether, mutagenesis studies suggest that Cl1 acts as an electrostatic cofactor that stabilizes the hydrogen-bond network around

the Mn_4CaO_5 complex and helps prevent inhibitory ion-pair formation, ensuring efficient turnover under physiological conditions [18].

Beyond steady-state catalysis, studies suggest that chloride plays an important role during photoassembly of the Mn_4CaO_5 cluster, particularly in the early light-driven steps when Mn^{2+} is oxidized to Mn^{3+} and proton removal is required. Cl^- addition to manganese-depleted PSII membranes accelerates photoactivation, shortens the lag phase, and more effectively restores O_2 evolution [88, 89]. Under low chloride conditions, an inverse H/D solvent isotope effect has been observed, indicating that proton removal at the high-affinity manganese-binding site becomes rate-limiting and that chloride reduces this barrier [66]. Cl^- is also suggested to promote Mn^{3+} formation during the first oxidation step, likely by stabilizing the hydrogen-bond network and enabling deprotonation events required to form μ -oxo/ μ -hydroxo bridges [88]. Models also suggest that chloride's presence modulates the local proton environment to facilitate these early deprotonation events, though the molecular mechanism remains under investigation [90].

4.4 Water channel function

Within PSII, several water-filled channels connect the Mn_4CaO_5 cluster to the lumen, including the Cl1 (broad) and O4 (narrow) channels, which have been proposed as major proton egress routes during S-state cycling based on crystallography and computational studies [91, 92]. These channels are partially hydrophobic pathways lined with protonatable residues and ordered waters, forming a highly organized hydrogen-bond network that appears to enable proton transfer through Grotthuss mechanisms while limiting bulk solvent intrusion [91–93]. The Cl1 channel leads past the mentioned Cl1 site between D1–D61 and D2–K317 toward the lumen, positioning chloride as a key regulator of channel integrity and modulating the protonation states of nearby residues to lower the energetic barrier for proton translocation [10, 11, 18, 69, 70, 85]. In this role, Cl^- synchronizes proton egress with the $\text{S}_2 \rightarrow \text{S}_3$ and $\text{S}_3 \rightarrow \text{S}_0$ transitions, sustaining efficient O_2 evolution [18]. Spectroscopic, computational, and mutagenesis studies determined Cl1 to be the primary proton egress pathway during these transitions, while the O4 channel is sometimes associated with proton release during the $\text{S}_0 \rightarrow \text{S}_1$ transition, varying by organism and PSII preparation [85, 91, 94]. Cl^- -dependent proton transfer was proposed to work alongside transient bicarbonate buffering at arginines near the Cl1 channel to neutralize excess charge, maintain electrostatic balance, and limit recombination under high-flux conditions [84, 95]. Although the precise role of Cl2 remains less defined, it is consistently observed in a pocket adjacent to the O4 channel [10, 70]. It has been proposed to help stabilize the hydrogen-bond network in this region, potentially influencing proton release in the early S-state transitions, but direct mechanistic evidence is still limited and its role remains an open question [18, 69].

4.5 Outstanding questions

Despite advances in understanding chloride's function in PSII, several key questions remain. Although Cl1 is widely accepted as a key proton egress site, the timing of proton release and its relationship to alternate routes such as the O4 channel are still debated. The functional significance of Cl2 is still unclear, with only

limited evidence for its involvement in proton transfer or water delivery. Resolving these roles will be essential for establishing a mechanism for proton management on the donor side of PSII.

5. Bicarbonate

5.1 Historical context

Among the inorganic cofactors of PSII, bicarbonate (HCO_3^-) has drawn attention because of its conserved binding site at the acceptor side and its still-debated mechanistic roles in facilitating electron transfer and proton management. Bicarbonate exists in equilibrium with dissolved CO_2 and plays both structural and regulatory roles [20, 96, 97]. It was first proposed as a direct participant on the donor side of PSII [98, 99], but while depletion experiments revealed photoinhibition and impaired electron transport [100, 101], the precise site and mechanism of action remained unclear. Govindjee and collaborators established the “bicarbonate effect” within PSII research [102]. Today, bicarbonate is firmly associated with the NHI (Figure 4) on the acceptor side of PSII [103–105], although donor side contributions have also been proposed [106], and several groups continue to actively research its role.

5.2 Acceptor side

5.2.1 Structural location

Bicarbonate is localized to the NHI on the acceptor side of PSII, positioned between the primary quinone electron acceptor Q_A and the secondary, exchangeable

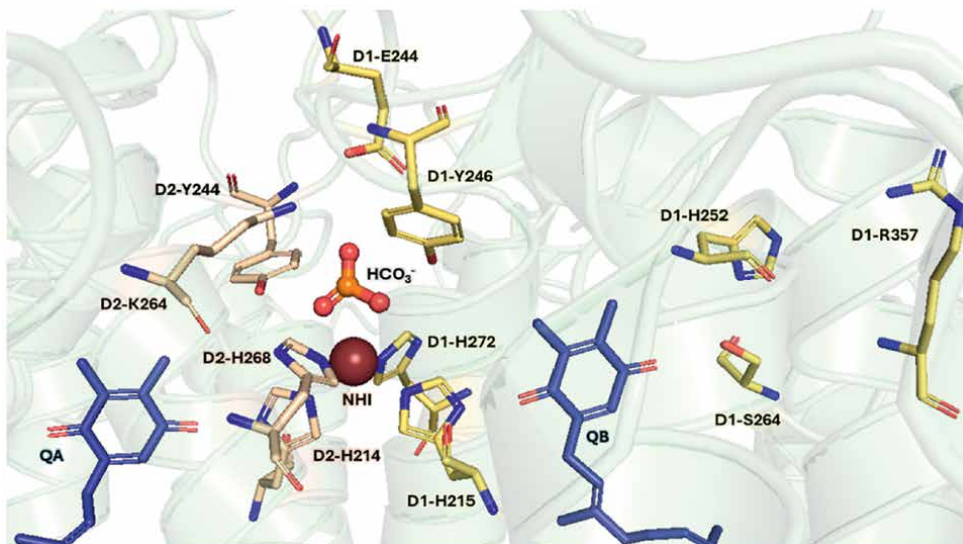


Figure 4.

The acceptor side of PSII, showing the quinones Q_A and Q_B (blue), the NHI (ruby) situated between them, and its coordinated bicarbonate ion (orange). Key residues from the D1 (light yellow) and D2 (beige) subunits which directly coordinate or interact with the NHI and the canonical bicarbonate site are shown. Additional residues relevant to protonation of the distal carbonyl of Q_B are shown at right.

quinone Q_B . Early spectroscopic studies provided indirect evidence for this placement. EPR signals from Q_A^- near the nonheme Fe^{2+} are perturbed by formate addition, suggesting that bicarbonate shares the same coordination sphere at the NHI [107]. FTIR spectroscopy revealed signatures of bicarbonate ligation, suggesting monodentate and bidentate coordination modes that depend on the oxidation state of the NHI [108]. Structural confirmation came from high-resolution crystallography of cyanobacterial PSII at 1.9 Å, which was followed by observations across cyanobacteria, algae, and higher plants, which consistently revealed bicarbonate coordinating to the NHI as a bidentate ligand [10, 11, 49, 75, 109, 110]. Nearby residues such as D1-R257 and D1-H252 may further anchor the bicarbonate through hydrogen bonding [111]. Further stabilization is provided by D2-Y244 and D1-Y246, which extend a hydrogen-bonding network and maintain the geometry of the bicarbonate ligand [10, 112]. Structural analysis of PSII assembly intermediates suggests that bicarbonate binding is dynamic during biogenesis. In partially assembled complexes, the NHI can instead be ligated by a glutamate from the D2 subunit [113, 114], but in the fully assembled PSII complex, this position is occupied by bicarbonate.

5.2.2 Functional roles

Beyond its structural placement at the NHI between Q_A and Q_B , bicarbonate fulfills several roles essential for PSII function. By coordinating with the NHI, bicarbonate stabilizes electron transfer between the primary and secondary quinones and fine-tunes redox potential at this site [19, 108, 115]. This coordination enhances efficiency of forward electron flow and minimizes charge recombination between $P680^+$ and Q_A^- [116]. In the absence of bicarbonate, O_2 can occupy this site and accept electrons, leading to superoxide formation. Although this provides a photoprotective outlet, it is less efficient and increases the risk of reactive oxygen species (ROS) damage [106, 117]. In addition, bicarbonate has been proposed to facilitate the protonation of Q_B to PQH_2 , although the extent of this contribution remains under investigation [118].

5.2.3 Mechanistic evidence

Early functional experiments provided the first mechanistic clues about bicarbonate's role on the acceptor side of PSII. Loss of HCO_3^- by CO_2 removal or displacement with formate consistently slowed Q_A^- reoxidation and impaired plastoquinone reduction, establishing a direct link between bicarbonate availability and electron-transfer efficiency [21, 115, 119]. Fluorescence decay patterns under HCO_3^- depletion resemble DCMU inhibition, indicating that the block was after Q_A but prior to the entry into the PQ pool [119, 120]. Herbicide-binding studies further revealed that atrazine affinity decreased under bicarbonate depletion, implicating its role at or near the Q_B site [120, 121]. Restoration of activity upon reintroduction of bicarbonate and partial rescue with small anions such as formate or acetate reinforced the view that the bicarbonate ligand at the NHI is directly coupled to the kinetics of Q_A to Q_B electron transfer [107]. Bicarbonate binding lowers the midpoint potential of Q_A/Q_A^- , favoring downstream electron transfer to Q_B and suppressing recombination with $P680^+$. Loss of bicarbonate shifts this potential to more positive values, slows Q_A^- to Q_B transfer, and increases the probability of charge

recombination, explaining elevated photodamage under bicarbonate depletion [116, 122].

Formate replacement introduces a pH-dependent conformational change that reduces protonation efficiency at Q_B , suggesting bicarbonate's dual role in electron transfer and proton delivery [123]. Anion substitution experiments further demonstrated that while ligands such as formate can coordinate the NHI, they fail to reproduce native electron-transfer kinetics, indicating that bicarbonate uniquely optimizes the energetics for plastoquinone reduction [20, 100, 105, 106]. Structural modeling and mutagenesis studies have suggested that residues near D1-H215, D1-H252, and D1-S264 form critical nodes of this pathway, with D1-Y246 providing an H-bond bridge between the HCO_3^- ligand and the Q_B pocket [124, 125]. In *Limnospira maxima*, depletion allows the first electron transfer to Q_B but blocks PQH_2 formation, associating either the NHI or the stromal surface near D1-H252 and D1-S264 as critical proton delivery sites [126]. This suggests that bicarbonate's role is not only redox tuning but also direct coordination of proton-coupled electron transfer for efficient PQH_2 turnover and photoprotection [127].

5.3 Donor-side effects

5.3.1 Structural role and location

While bicarbonate is well established as a ligand to the NHI on the acceptor side of PSII, its structural role on the donor side remains unresolved. In contrast to chloride, which occupies defined proton egress channels, high-resolution structural studies showed no evidence of a fixed bicarbonate binding site on the donor side [11, 75, 109, 110]. Instead, biochemical and spectroscopic studies suggest more transient or dynamic associations. Several studies support the idea that bicarbonate can associate dynamically with positively charged residues, particularly arginine and lysine, throughout PSII. Notably, CP43-R357, located in the second coordination shell of the WOC, has been highlighted as a potential interaction site. Its substitution leads to a decrease in O_2 evolution dependent on bicarbonate concentration [52, 128].

5.3.2 Functional and mechanistic roles during catalysis

Although no stable donor-side binding site for bicarbonate has been resolved structurally, functional studies suggest a mechanistic contribution to PSII catalysis [96, 129]. Bicarbonate may serve as a mobile proton carrier and buffer, diffusing through hydrogen-bond networks to neutralize protons released during water oxidation [100, 130]. This role complements that of Cl^- , which helps define static proton egress pathways, whereas bicarbonate extends buffering capacity under conditions of high proton release rates or changed hydrogen bonding [66, 89]. HCO_3^- restoration of normal S-state cycling in the D2-K317A mutant but not in D1-D61A or CP43-R357K supports the idea that proton removal is mediated by a diffusible base such as bicarbonate rather than a fixed residue [128]. Bicarbonate improves O_2 evolution under high light and rescues activity in hydrogen-bond network mutants [100, 130]. Beyond its catalytic role, HCO_3^- has been linked to photoassembly of the Mn_4CaO_5 cluster. During photoactivation, stepwise addition of Mn^{2+} ions and oxidation are coupled to proton release, requiring local buffering to stabilize the high energy intermediate states [131, 132]. Bicarbonate is proposed to associate with

positively charged residues near the donor side, electrostatically steering Mn^{2+} toward the apo-WOC site and thus enhancing yield and rate of assembly [133]. Isotopic tracing using ^{13}C -labeled bicarbonate provided direct evidence of transient coordination to Mn^{2+} during the initial photoassembly steps, stabilizing Mn^{3+} and facilitating succeeding metal ion incorporation [134]. Depletion of bicarbonate inhibits O_2 evolution recovery after photodamage, while reinserting bicarbonate reestablishes efficient photoactivation [106]. Together, these findings indicate that bicarbonate serves dual roles on the donor side: (1) buffering of protons during water oxidation to prevent charge accumulation; and (2) enabling photoassembly and repair of the WOC by stabilizing Mn intermediates and promoting their correct positioning.

5.4 Outstanding questions

Although bicarbonate's role at the NHI is well known, uncertainties remain regarding whether it behaves exclusively as a bound cofactor or also as a mobile, diffusible cofactor. Functional studies suggest that bicarbonate can buffer WOC proton release through hydrogen-bond networks [100, 130, 131], but structural evidence has localized it to the acceptor site [10, 11]. Species variation adds complexity; for instance, *Limnospira maxima* lives in extremely high concentrations of bicarbonate and exhibits unusual PSII behavior, raising the possibility that bicarbonate plays a distinct mechanistic role in an adapted organism [106, 128]. Structurally, donor-side effects remain unresolved: No structure has resolved bicarbonate near the WOC [10, 11, 110], and standard purification protocols may unintentionally deplete weakly bound bicarbonate [99]. While studies have associated bicarbonate in photoassembly and repair of the Mn_4CaO_5 cluster [23, 132], current evidence is restricted by assays and the inability to determine short-lived intermediates. Addressing these limitations requires integrative approaches combining structures, spectroscopy, and organism-specific studies to elucidate how bicarbonate supports catalysis and biogenesis.

6. Nonheme iron

6.1 Structure and function overview

A mononuclear NHI is located between the Q_A and Q_B quinones on the acceptor side of PSII. NHI is a hexacoordinate Fe^{2+} that has a distorted octahedral geometry, coordinating the bicarbonate ion discussed in the previous section and four histidines: D1-H215, D1-H272, D2-H214, and D2-H268. D2-H214 and D1-H215 are positioned between the NHI and Q_A and Q_B , respectively, forming hydrogen bonds to their respective quinones [10, 19]. Each of these four histidines interacts via an unprotonated nitrogen in the imidazole ring as a monodentate ligand, while the structural bicarbonate stromal to the NHI is a bidentate ligand via two of its oxygens [19]. NHI mediates electron transfer between Q_A and Q_B , but does not normally play a direct redox role [19]. However, strong oxidants may convert NHI to iron(III) [135], which then accepts electrons from Q_A^- to return to ground state, leading to observations of two-electron removal from the WOC on a single flash [136]. This phenomenon appears to be limited to conditions under which the structural

bicarbonate ligand or similar anions are unavailable [137]. NHI's redox midpoint potential is relatively high, ~360 mV at pH 7.8 in the PSII reaction center [138]. Studies showing pH dependence suggest coupling to nearby protonatable residues [139]. NHI's presence is needed to maintain geometry and electrostatic environment between cofactors including pheophytin and Q_A [19]. NHI has been posited to play a role in ROS detoxification when redox-active [140]. Removal of Fe^{2+} from *T. elongatus* PSII does not impede electron transfer from pheophytin to Q_A [141], excluding a role of NHI at this step. However, EPR and ENDOR spectroscopy following Fe^{2+} depletion [142] determined that Q_A^- is not magnetically coupled to Fe^{2+} . Zn^{2+} has been shown to occupy the NHI site and restore native-like structural, if not functional, features, while copper can similarly mimic the structure but is redox-active in the site [143].

6.2 Role in photoprotection

NHI has historically been viewed as a participant in photoprotective mechanisms. When charge recombination occurs between reduced Q_A^- and oxidized $P680^+$, singlet oxygen, a ROS implicated in photodamage, can be formed as a by-product. Redox potential and stability of Q_A^- , which are regulated by NHI and its bicarbonate ligand, influence recombination probability. The redox potential of NHI decreases across pH 6–8, showing sensitivity to protonation in the environment [138]. Ishikita and Knapp proposed that pH dependence is due to coupling to a network of protonatable residues near NHI that could stabilize Q_A^- and reduce the rate of recombination with $P680^+$ [139]. This buffering effect was proposed to delay harmful back reactions and decrease singlet oxygen formation, especially when forward electron transfer to Q_B is impaired. NHI would thus modulate the flow of electrons. Bicarbonate availability and identity of the D1 protein isoform influence the redox potential of NHI [144]. These may be modulated under stress conditions, since maintaining redox control on the acceptor side is necessary for photoprotection.

6.3 Open questions

While the two-electron gate mechanism of electron transfer through Q_B is established, the protonation steps remain a subject of study. Structural data suggest two-proton delivery pathways, one involving D1-H215 and D1-Y246 for the Q_B carbonyl proximal to the NHI, while the other involves D1-S264 and D1-H252 near the distal site [19]. Bicarbonate stabilizes these networks, but it is not clear if it directly delivers protons [124, 126]. Manipulations on the donor side, such as Ca^{2+} depletion or Sr^{2+} substitution, shift Q_A redox properties. This suggests that there is a long range coupling between Mn/Ca proton dynamics and acceptor-side redox tuning [145]. Such interactions suggest that there may be an integrated electrostatic network, yet the exact mechanistic links are unknown.

7. Conclusion

While the inorganic cofactors of PSII have been known for decades, their functions remain incompletely understood and specific significant questions remain for each. Regarding the manganese and calcium that make up the WOC, targets of

future research include the specific mechanism of water incorporation, the composition of the S₄ state, and the precise oxidation states of manganese that are needed to form molecular oxygen. Cl1 is comparatively well understood, but the specific mechanism of proton egress it facilitates remains unknown and there is still no clear evidence for the role of highly conserved Cl2. Bicarbonate's role at the NHI is quite clear, but its donor-side site activity is still unknown and its role in protonation on the acceptor side appears to extend beyond the known NHI site. Finally, the extent of redox activity of the NHI *in vivo* remains to be determined. Obtaining answers to these questions will allow for improved efficiency of conversion of light to human-available energy in a range of applications.

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Genetic Modifications in Cyanobacteria: Current Status and Future Prospects

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Abstract

Cyanobacteria are powerful model systems for fundamental and applied research, ranging from photosynthesis and nitrogen fixation to biofuel and biomolecule production. Over the past five decades, the field of cyanobacterial genetics has transformed from basic mutagenesis and plasmid transfer methods into a sophisticated synthetic biology discipline supported by advanced molecular and computational tools. Early developments, such as the discovery of natural competence, conjugative DNA transfer, and shuttle vector construction, established the foundation for stable transformation systems in diverse strains. The subsequent identification of strong native and heterologous promoters, synthetic ribosome binding sites, and optimized selection markers further refined the control of gene expression. Genome sequencing of cyanobacterial strains enabled vector design and genome-scale metabolic engineering. More recently, the advent of markerless selection systems and CRISPR/Cas-based genome editing tools has revolutionized the precision and efficiency of genetic manipulation in cyanobacteria. This chapter provides an integrated overview of the history, evolution, and applications of genetic tools in cyanobacteria. It uniquely covers progress from classical recombination to CRISPR technologies and the emerging future consisting of AI-driven gene design, multiomics integration, and minimal chassis development, while highlighting challenges in achieving standardized, stable, and scalable cyanobacterial strains for sustainable biotechnology.

Keywords: CRISPR/Cas, cyanobacteria, genetic manipulation, genetic tools, markers, plasmids, promoters, ribosome-binding sites, riboswitches, shuttle vectors

1. Introduction

Genetic modification can be defined as the deliberate alteration of a cell's or an organism's DNA content in order to produce a desired trait for applications in the fields of medicine, research, and agriculture [1]. Humans have been performing genetic manipulations for thousands of years by selectively breeding organisms with desired traits (e.g., the domestication of dogs, cabbage, and chicken) [2]. In modern times, it relies on sophisticated molecular techniques to add, overexpress, or silence

gene sequences in an organism to achieve a targeted phenotype or product in a sustainable way [1].

In this context, cyanobacteria are promising candidates for sustainable biotechnology because they efficiently use sunlight and CO₂ to produce diverse metabolites, require simple nutrients, and are genetically easy to modify [3]. Genetic modification in cyanobacteria offers a versatile platform for the sustainable production of biomass and renewable biofuels, including ethanol, butanol, biodiesel, and hydrogen [4–7]. Beyond energy applications, engineered cyanobacteria hold significant promise in environmental and industrial biotechnology. They can be used for bioremediation of heavy metals and other pollutants, biofertilization to enhance soil nutrient content, and aquaculture to improve water quality and support feed production [8]. Additionally, they can serve as biofactories for the synthesis of biologically active molecules and high-value compounds, such as vitamins, pigments, antioxidants, and pharmaceuticals. This capability helps bridge the gap between sustainable production and commercial application [9–12].

From the discovery of the DNA double helix by Watson and Crick in 1953 [13] to the recently discovered and emerging technologies like CRISPR-Cas [14], the field of genetic engineering has seen a series of transformative discoveries that have made genetic manipulation easy and quick. So far, genetic engineering has been implemented in a wide range of organisms, ranging from bacteria to humans, but the availability and advancement of genetic engineering tools are not the same across different species [15]. Simple, well-characterized organisms like bacteria and yeast, with less complex genomes, have robust and well-established genome editing tools, while complex organisms like cyanobacteria continue to present challenges. The gene editing techniques discovered for heterotrophic bacteria, such as *Escherichia coli*, cannot be directly implemented on cyanobacteria due to differences in the cellular organization and physiology of these organisms [15]. The differences that make cyanobacterial systems challenging include the presence of thylakoid membranes, mucilaginous sheaths [16], specialized cells like heterocysts and akinetes, along with multiple genome copies per cell (polyploidy), which complicate genetic segregation and stable mutant generation [17–19]. In addition, many cyanobacteria possess strong restriction–modification systems that degrade foreign DNA, making transformation inefficient [20–22]. They also possess a very efficient recombination machinery [23], which not only facilitates the integration of exogenous DNA by homologous recombination but can also result in the unintended integration of replicative vectors, making mutant behavior unpredictable. Finally, the presence of circadian rhythms [24] can lead to time-dependent behavior in engineered genetic networks.

Furthermore, standardized genetic parts, such as promoters, ribosome-binding sites (RBSs), and terminators, often behave unpredictably in cyanobacteria because their transcriptional and translational machinery, light-regulated gene expression, and regulatory networks differ from those of model bacteria [25, 26]. Therefore, these regulatory elements must be characterized and optimized within cyanobacterial systems for consistent and predictable gene expression outcomes.

Nevertheless, the rapid expansion of genome sequencing and annotation data for diverse cyanobacterial species over the past few decades has greatly facilitated the development of molecular tools for their genetic manipulation. Transfer of exogenous DNA into unicellular or filamentous strains has been made easy using

methods like natural transformation, electroporation, and conjugation with self-replicating plasmids or integrative vectors. A large number of positive and negative selection markers, along with reporter constructs, have also been established for promoter activity measurements and protein localization studies. Moreover, extensive knowledge of restriction–modification systems in several cyanobacterial strains has been acquired to improve transformation efficiency [27]. The advancement of genetic tools, including metabolic engineering, synthetic biology, and CRISPR-based genome editing, has significantly expanded the potential of cyanobacteria, allowing precise manipulation of metabolic pathways to enhance productivity, stress tolerance, and environmental adaptability [28]. In this chapter, we are going to summarize the history, evolution, and existing knowledge of genetic manipulation tools in cyanobacteria, along with their applications and future prospects.

2. History and evolution of genetic manipulation tools in cyanobacteria

The discovery of the DNA double helix [13], followed by the identification of ligases, restriction enzymes, and plasmid cloning systems [29–32], laid the foundation of recombinant DNA technology. This was later revolutionized by the establishment of the polymerase chain reaction in the 1980s [33]. While genetic engineering was advancing across diverse systems, cyanobacterial genetic manipulation tools also gradually evolved from the 1970s through the 1990s, building the foundation for modern synthetic biology applications. Early studies include the first experimental report demonstrating natural genetic transformation (i.e., uptake and incorporation of exogenous DNA) in a cyanobacterium (*Anacystis nidulans*, now known as *Synechococcus elongatus* PCC 6301), among others [34]. Another study revealed that many cyanobacterial strains carry circular plasmid DNAs (CCC DNAs) with inter-strain homologies and potential for horizontal transfer, suggesting the possibility for the development of plasmid-based cloning tools [35]. Simultaneously, classical genetic selections and mutant screens, like amino acid antimetabolites for regulatory mutants, were being established, providing early genetic systems for cyanobacteria [36].

In the 1980s and early 1990s, cyanobacterial genetic manipulation research focused on establishing DNA transformation methods while developing shuttle vectors, selectable markers, and transposon systems for mutagenesis. The first successful attempts to introduce foreign DNA into cyanobacteria through natural competence, conjugation, and electroporation were reported [37–39, 40]. These classical approaches were complemented by transposon mutagenesis with transposon systems such as Tn5 and Tn9, which allowed random gene disruption for functional analysis [41–43]. Site-directed mutagenesis for precise gene substitution by homologous recombination was also established [44, 45]. Complementation assays were developed to confirm gene function by recovering mutant phenotypes with wild-type copies [46, 47].

A major breakthrough came with the introduction of conjugation between *Escherichia coli* and cyanobacteria, mediated by broad-host-range IncP plasmids such as RP4. This enabled efficient gene transfer across diverse unicellular and filamentous cyanobacteria [37, 48, 49]. Electroporation soon followed as a simpler and more controlled alternative for introducing DNA [39, 50]. However,

restriction–modification systems in cyanobacteria often degraded unmethylated foreign DNA, reducing transformation efficiency [38, 51]. To address this, shuttle vectors were developed with dual replication origins for *E. coli* and cyanobacteria [51–53]. Among these, IncQ-derived plasmids such as RSF1010 exhibited wide host range and stability, functioning efficiently in several species [54–56].

Around this time, selectable markers, such as antibiotic resistance genes conferring tolerance to neomycin, erythromycin, spectinomycin, chloramphenicol, and kanamycin, were widely used. Hybrid cassettes combining native cyanobacterial promoters with bacterial resistance genes improved expression and selection efficiency in both unicellular and heterocystous strains [57, 58].

The early 1990s marked a milestone with promoter–reporter fusion systems that allowed in vivo monitoring of gene regulation. Reporters such as lacZ (β -galactosidase) and luxAB (luciferase) were used to quantify promoter activity [59, 60]. Strong constitutive promoters such as *PpsbA2*, *PcpcB*, and phage-derived promoters like λ PR and λ PL were identified for high-level gene expression [48, 61, 62]. Regulatable promoters, including *Ptac* (LacI-controlled) and phage T7 promoters, which were an early attempt at orthogonal expression, were also reported, but their use was limited due to challenges like inducer cost and heat sensitivity [63, 64]. Nevertheless, these systems paved the way for modern synthetic promoters.

An important turning point came with the mapping and sequencing of cyanobacterial genomes in the early 1990s [65]. By 1996, the complete genome sequence of *Synechocystis* sp. PCC 6803 was published, marking the first photosynthetic organism to be fully sequenced and revolutionizing the field by providing genetic blueprints [66]. Shortly after this, the CyanoBase database was established [67] to add and annotate cyanobacterial genomic information. Genome sequencing of different cyanobacterial strains enabled the identification of neutral integration sites, native promoters, and restriction–modification systems, accelerating the design of vectors, promoters, and other transcriptional and translational entities, as discussed in the following sections.

Since the 2000s, the cyanobacterial genetic toolkit has expanded rapidly with optimized shuttle vectors, modular cloning systems (BioBrick, CYANO-VECTOR, CyanoGate, etc.) [68–70], engineered promoters and RBS libraries [71], inducible systems adapted to cyanobacterial physiology, and the onset of CRISPR-based editing. CRISPR–Cas technology was adapted in cyanobacteria in 2014, first demonstrated in *Synechococcus elongatus* PCC 7942 [72]. Subsequent advancements enabled efficient, markerless genome editing [73] and transcriptional regulation through CRISPR interference (CRISPRi) [74, 75], followed by Cas12a-based and multiplexed CRISPRi/a systems that now support genome-scale manipulation across species [76]. This period also witnessed a surge in the application of genetically engineered cyanobacteria for industrial and biotechnological purposes, such as biofuel, bioplastic, and high-value compound production. Additionally, they served as model systems to elucidate fundamental biological processes, including gene function, photosynthetic regulation, and transcriptional control [6, 77–79].

In summary, cyanobacterial genetic manipulation evolved from classical microbiological genetics and recombinant-DNA techniques in the 1970s, through shuttle vectors and reporter systems in the 1990s–2000s, to the modern synthetic biology and CRISPR era of today, as shown in **Figure 1**.

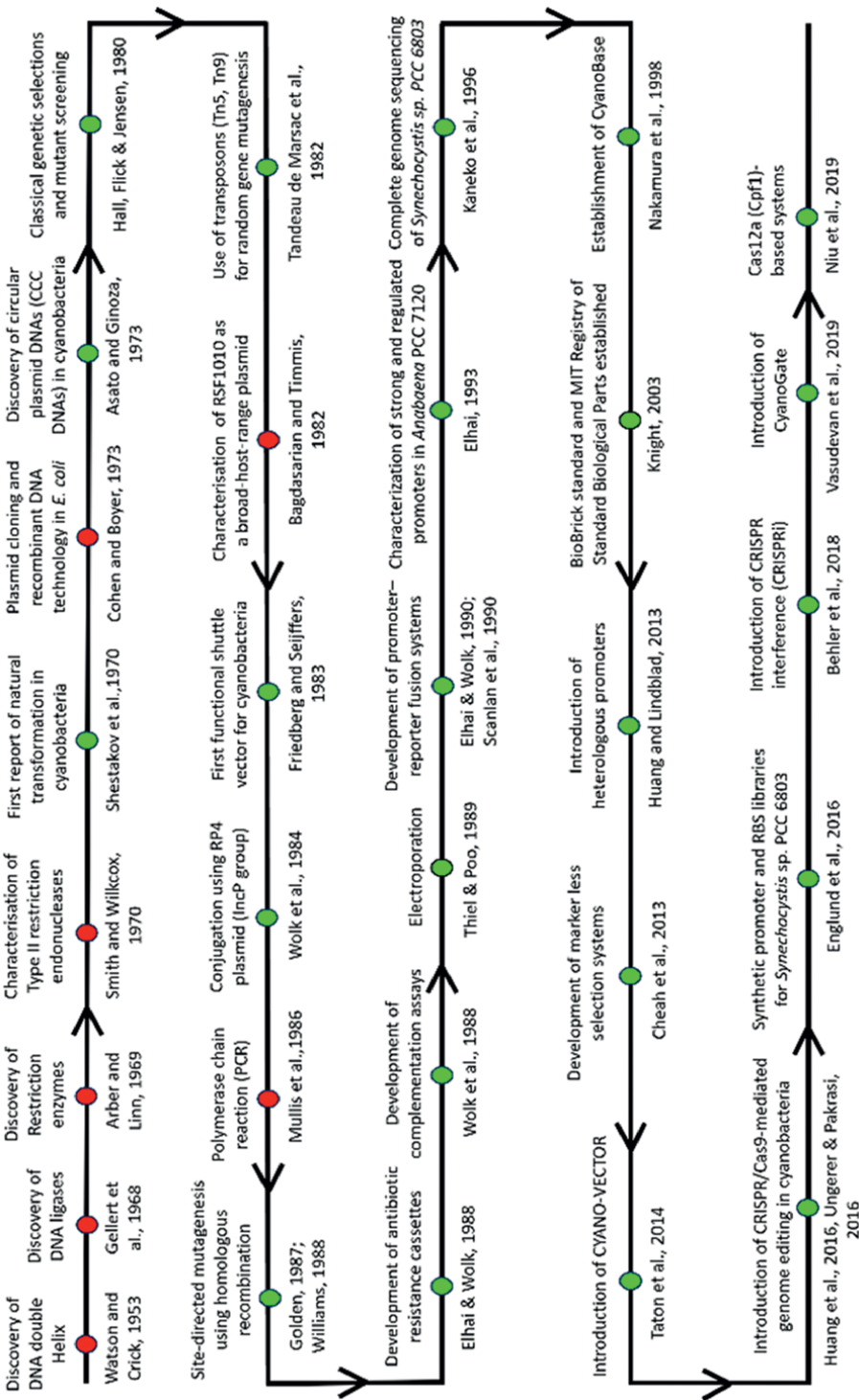


Figure 1. Chronological overview of key events in the progress of cyanobacterial genetic manipulation (1970–2020). The red points show some of the general milestones that laid the foundation of genetic engineering [13, 29–33, 54] while the green points are key events specific to cyanobacteria [26, 34–39, 41, 44–46, 52, 59–61, 66–76].

3. Genetic tools for cyanobacteria

Effective genetic modification of cyanobacteria requires some basic tools, called genetic tools, that help in controlling gene expressions and post-translational regulations. Many genetic tools, like neutral sites, integrative or shuttle vectors, promoters, ribosome binding site (RBS) sequences, riboswitches, markers, CRISPR/Cas systems, etc., have been developed for both basic biological research and strain engineering in cyanobacteria. These tools have been successfully used in studies involving gene transfer, gene expression, gene control, and metabolic modification [80–82]. Currently, the development and application of genetic tools in cyanobacteria are largely lagging behind the toolboxes widely available for *E. coli*, *Bacillus subtilis*, and *S. cerevisiae* due to the earlier-mentioned challenges [15]. In this section, we discuss some of the available genetic tools in cyanobacteria, their current status, and limitations.

3.1 Neutral sites

Heterologous gene insertion in cyanobacteria mainly relies on transformation and homologous recombination, for which researchers often use neutral chromosomal sites that do not affect host physiology (e.g., *slr0168* [81], *psbA1* in *Synechocystis* [83], NSI/NSII in *S. elongatus* 7942 [84], and *glpK* in *Synechococcus* 7002 [85]). A detailed list of neutral sites in *Synechocystis* and other cyanobacterial strains can be found in the literature [86, 87]. However, the number of known neutral sites is limited, and transformation efficiency in such cases depends on natural competence, which makes this method restricted to established model cyanobacteria [88]. Furthermore, the polyploidy of species like *Synechocystis* makes complete segregation laborious and time-consuming [19], limiting the flexibility and speed of chromosomal integration for complex genetic engineering.

3.2 Replicative plasmids and shuttle vector systems in cyanobacteria

Replicative plasmids are extrachromosomal DNA elements that replicate autonomously, either through host-encoded proteins or their own replication machinery. Unlike chromosomal integration, plasmid-based systems avoid segregation issues in polyploid cyanobacteria and thus enable faster genetic manipulation [87]. Many native plasmids confer traits such as antibiotic resistance or stress tolerance [89] and vary in size, replication origin, and copy number, ranging from low (2–10 copies) to high (>20–100 copies) per cell. Synthetic plasmids often include selectable markers and multiple cloning sites; however, conventional *E. coli* vectors, such as pUC [90], do not replicate in cyanobacteria, leading to the need for shuttle vectors.

Among the few broad-host-range shuttle vectors, derivatives of RSF1010 are the most extensively used and can replicate in over 30 Gram-negative bacteria, including *Synechocystis*, *Synechococcus*, and *Anabaena* [69, 91]. Several optimized RSF1010 derivatives, such as pPMQAK1, pSL1211, pFC1, pDF, pSHDY, and the pSEVax5n series, have been developed to reduce size and improve stability. Another broad-host-range plasmid, RK2, also replicates in *Synechocystis* [92]. Since only one plasmid of the same incompatibility group can stably coexist in a host, synthetic shuttle vectors have been developed by fusing *E. coli*-compatible replication systems with

Endogenous/native plasmid and host strain	Shuttle vector or shuttle vector series	Reported host strains	References
pCA2.4 (<i>Synechocystis</i> sp. PCC 6803)	pCA-UC118, pSOMA series	<i>Synechocystis</i> sp. PCC 6803, <i>Nostoc</i> sp. PCC 7120	[95, 96]
pCB2.4 (<i>Synechocystis</i> sp. PCC 6803)	pCB-SC101, pSOMA series	<i>Synechocystis</i> sp. PCC 6803	[95, 96]
pCC5.2 (<i>Synechocystis</i> sp. PCC 6803)	pSCB series, pCCM1-FbFP, pYS series	<i>Synechocystis</i> sp. PCC 6803, <i>Synechococcus elongatus</i> PCC 7942, <i>Synechococcus</i> sp. PCC 7002, <i>Nostoc</i> sp. PCC 7120	[97–99]
pSYSA (<i>Synechocystis</i> sp. PCC 6803)	VIII series	<i>Synechocystis</i> sp. PCC 6803, <i>Synechococcus elongatus</i> PCC 7942	[100]
pSYSX (<i>Synechocystis</i> sp. PCC 6803)	p6031 and p6090 series	<i>Synechococcus elongatus</i> PCC 7942	[101]
pANS/pUH24 (<i>Synechococcus elongatus</i> PCC 7942)	pLS103, pSG111, pAM series	<i>Synechococcus elongatus</i> PCC 7942, <i>Nostoc</i> sp. PCC 7120	[101–104]
pAQ1 (<i>Synechococcus</i> sp. PCC 7002)	pAQE series	<i>Synechococcus</i> sp. PCC 7002	[105]
pDU1 (<i>Nostoc</i> sp. PCC 7524)	pRL series, pRL2697, pRL25C, pDUCA7, pRL489, pRL1050	<i>Nostoc</i> sp. PCC7120, <i>Nostoc</i> sp. PCC 7524, <i>Anabaena</i> sp. M-131, <i>Fischerella muscicola</i> PCC 7414 and <i>Chlorogloeopsis fritschii</i> PCC 6912, <i>Chroococcidiopsis</i> spp. str. 029, 057, and 123, <i>Oscillatoria</i> MKU 277, <i>Fischerella thermalis</i> SAG 4.84	[37, 46, 61, 64, 106–110]
pDC1 (<i>Nostoc</i> sp. MAC PCC 8009)	pSCR119/202, pSUN119/202	<i>Nostoc punctiforme</i>	[111, 112]
pGL3 (<i>Plectonema boryanum</i> sp. PCC 6306)	pPBH201	<i>Nostoc</i> sp. PCC 7120, <i>Plectonema boryanum</i> sp. PCC 6306	[113]
pFDA (<i>Fremyella diplosiphon</i>)	pJCF series	<i>Fremyella diplosiphon</i>	[114]

Table 1.
List of shuttle vectors derived from endogenous/native plasmids of cyanobacteria and their reported host strains (apart from *E. coli*).

cyanobacteria-specific replication elements. These vectors function in both unicellular species (*Synechocystis*, *S. elongatus* PCC 7942, *Synechococcus* PCC 7002) and filamentous strains like *Anabaena* sp. PCC 7120, which are difficult to transform through homologous recombination [87]. Nevertheless, mechanistic understanding of plasmid replication, copy number regulation, and maintenance in cyanobacteria remains incomplete [93]. A comprehensive list of cyanobacterial shuttle vectors, along with their host strains, is given in **Table 1**.

In parallel, modular cloning systems have been designed to standardize cyanobacterial genetic engineering. The CYANO-VECTOR platform [69] and

CyanoGate [70], based on Golden Gate MoClo standards, facilitate rapid assembly of multigene constructs compatible with several hosts [94]. These systems, together with the increasing availability of endogenous plasmid-derived shuttle vectors, have significantly expanded the cyanobacterial genetic toolbox, enabling modular, high-level gene expression and advanced synthetic biology applications across diverse cyanobacterial species.

3.3 Promoters

For successful heterologous gene expression in cyanobacteria, it is not only essential to introduce and maintain foreign genes but also to ensure their optimal expression for achieving a high yield of targeted products. This is achieved by promoter engineering. Promoter engineering is a very effective and frequently used tool for controlling gene expression. Promoters are specific DNA sequences that recruit RNA polymerase and initiate transcription. They can be classified based on origin – endogenous (produced from the host) or heterologous promoters (produced from another organism), and activity – constitutive (which are always active) or inducible promoters (which require an additional regulatory element) [87]. A comprehensive list of commonly used promoters, their inducers (in the case of inducible promoters), and host strains has been given in **Table 2**.

- Endogenous or native promoters

Endogenous promoters, or native promoters such as photosystem promoters, have been extensively studied for heterologous gene expression in cyanobacteria, but most exhibit light-dependent activity and limited predictability,

Inducible promoters			
Name	Inducer	Host strains	References
PL03	aTc ligand	<i>Synechocystis</i> 6803	[26]
Pscs6-2	IPTG	<i>Synechocystis</i> 6803	[115]
PO2	FNR under dark conditions	<i>Synechocystis</i> 6803	[116]
PompC	Envz under dark conditions	<i>Synechocystis</i> 6803	[117]
Pvan	Vanillate	<i>Synechococcus</i> 7942	[118]
PBAD	L-arabinose	<i>Synechococcus</i> 7942, <i>Synechocystis</i> 6803	[117, 119]
PrhaBAD	Rhamnose	<i>Synechocystis</i> 6803	[120]
PisiAB	Fe ²⁺	<i>Synechocystis</i> 6803, <i>Synechococcus elongatus</i> 7942, <i>Anabaena</i> , and <i>Leptolyngbya</i> BL0902	[69, 81, 121]
PphoA	Inorganic phosphorus	<i>Synechocystis</i> , <i>Synechococcus elongatus</i> sp. PCC 7942, <i>Anabaena</i> , and <i>Leptolyngbya</i> BL0902	[69]
PnrsB	Ni ²⁺ or Co ²⁺	<i>Synechocystis</i>	[95, 122–126]
PpetE	Cu ²⁺	<i>Synechocystis</i> , <i>Anabaena</i>	[71, 127–133]

Inducible promoters		
Constitutive promoters		
Name	Host strains	References
PR-PS	<i>Synechococcus</i> 7942	[134]
PcpC560	<i>Synechocystis</i> and <i>S. elongatus</i> UTEX2973	[70, 135–138]
PpsbA	<i>Synechocystis</i> , <i>S. elongatus</i> 7942, <i>Anabaena</i> , and <i>Synechococcus</i> 7002	[61, 139–141]
PpsbA1	<i>S. elongatus</i> 7942, <i>Synechocystis</i> , <i>Anabaena</i> , and <i>Leptolyngbya</i> BL0902	[4, 69, 142–144]
PpsbA2S	<i>Synechocystis</i>	[71, 139, 145–147]
PA2520	<i>Synechococcus</i> 7002	[148]
PA2579	<i>Synechococcus</i> 7002	[148]
Plac	<i>Synechococcus</i> 2973	[149]
Ptrc	<i>Synechococcus</i> 2973	[150]
BioBrick BBa_J23101	<i>Synechocystis</i> , <i>S. elongatus</i> 7942, and <i>Anabaena</i>	[26, 71, 72, 95, 128, 151]
BioBrick BBa_J23119	<i>Synechocystis</i> , <i>S. elongatus</i> 7942, <i>Synechococcus</i> 7002, <i>Anabaena</i> , and <i>S. elongatus</i> UTEX2973	[70–72, 74, 136, 152, 153]
PconII	<i>Synechocystis</i> , <i>S. elongatus</i> 7942, <i>Synechocystis</i> sp. Strain WHSyn, <i>Anabaena</i> , and <i>Leptolyngbya</i> BL0902	[69, 72, 92, 154]
Prbc	<i>Synechocystis</i>	[71, 129, 146, 155]
PrnpB	<i>Synechocystis</i>	[71, 128, 155]
Ptrc1O	<i>Synechocystis</i>	[26, 127, 128, 139, 155]

Table 2.
 A list of promoters developed for genetic engineering in cyanobacteria.

making them less suitable for metabolic engineering [3, 142, 156, 157]. Most of these promoters are derived from genes related to photosynthesis, such as CO₂ fixation (*PrbcL*, *Pcmp*, *PsbT*), photosystem I (PSI; *PpsaA*, *PpsaD*), PSII (*PpsbA1*, *PpsbA2*), and the photosynthesis antenna protein phycocyanin (*PcpC*) [158]. Studies in *Synechococcus* PCC 7002 involving the expression of heterologous yellow fluorescent protein (YFP) showed that most native promoters yielded fluorescence similar to background wild-type fluorescence [148], indicating that native promoters require additional design to achieve the desired expression of heterologous genes [3]. Similarly, experiments conducted on *Synechococcus* PCC 7942 to increase the expression of limonene synthase for the production of limonene revealed that only one native promoter among all tested promoters produced as much limonene as the heterologous *Ptrc* promoter, while

a heterologous *pea psbA* promoter achieved the highest expression [159]. This is because native promoters often contain unknown regulatory elements that can cause unpredictable gene expression and genetic instability, where strains suddenly drop in productivity or performance [160]. Such issues are further complicated by the polyploid nature of cyanobacterial chromosomes, making full segregation difficult and leading to variability in productivity. These findings emphasize the need for more tightly regulated, well-characterized heterologous promoters and genetic parts to ensure stable and predictable gene expression in cyanobacteria [3].

- Heterologous promoters

Heterologous promoters have also been explored in cyanobacteria to overcome native regulatory constraints and avoid strain instability from repeated sequences. Common *E. coli* promoters, such as λ PL, λ PR, and *PLac*, show little to no activity in *Synechocystis* PCC 6803, with minimal reporter fluorescence detected from *Plac*, *PR*, *P_{trc}*, and *P_{tet}* [155]. Modifying elements like the -10 region to the consensus sequence (TATAAT) can improve expression in *E. coli* but not in cyanobacteria [26], because fully consensus promoters may reduce activity due to excessive contact between RNA polymerase and the promoter [161]. Promoter performance also varies between cyanobacterial strains. For example, in *S. elongatus* sp. PCC 7942, heterologous promoters *P_{trc}*, *PLlacO1*, *PconII*, *PJ23101*, and *PJ23119* were tested, with *PconII* and *PJ23119* giving the highest expression [72].

Heterologous promoters behave unpredictably in cyanobacteria due to fundamental differences in their transcription machinery compared to model bacteria like *E. coli*. Cyanobacterial promoters generally have a strong -10 element (TATAAT) but lack a well-defined -35 consensus, suggesting reliance on sigma factors for promoter recognition [162]. Promoters in cyanobacteria are classified into three types: Type I (σ 70; contains both -10 and -35), Type II (-10 plus activator-binding sites, e.g., *glnB* P2 regulated by *NtcA*), and Type III (lacking consensus elements; stress-responsive) [128]. Sigma factors are grouped into Group 1, primary (*sigA*); Group 2, nonessential/environment-responsive (*sigB*, *sigC*, *sigD*, *sigE*); and Group 3, stress-specific (*sigF*, *sigG*, *sigH*, *sigI*) families, but limited understanding of their interactions hampers prediction of promoter activity [163, 164]. Further differences in RNA polymerase structure, sigma factor specificity, and overall transcriptional regulation have been extensively described in the literature [3]. These variations help explain why heterologous promoters frequently exhibit altered or inconsistent performance in cyanobacteria compared to *E. coli*.

- Inducible promoters

Inducible promoters are crucial tools in cyanobacterial genetic engineering, as they allow precise control over gene expression. Ideally, such promoters show minimal basal activity, respond rapidly to inducers, and exhibit tunable expression proportional to inducer concentration [93]. They are particularly valuable when the introduced genes cause a metabolic overload or produce toxic byproducts [165]. Native inducible promoters in cyanobacteria respond to various environmental or metal ion cues. Examples include the nickel- and cobalt-inducible *P_{nrsB}* [71], the copper-responsive *P_{petE}* and *CoaA* [127], the

zinc-responsive *Psm*, the nitrate/nitrite-inducible *PnirA*, and the light-regulated *PpsbA* [154]. Among these, *PnrsB* is one of the most effective, providing tight regulation and strong induction upon exposure to Ni^{2+} or Co^{2+} [71]. However, many native metal-inducible systems face limitations, such as inducer toxicity at higher concentrations and interference with cellular metabolism [71, 127, 155, 165, 166].

Several heterologous induction systems from *E. coli*, like the IPTG-inducible *P_{trc}* [83, 167], *P_{tac}* [61], and *P_{LlacO1}* [6], have also been tested in different cyanobacteria, with reports of both strong and poor expression depending on the host strain and construct design. To expand the toolbox, new heterologous systems, like the anhydrotetracycline (aTc)-inducible *P_{L03}* promoter [26] and sugar-inducible systems such as *P_{BAD}* (L-arabinose-inducible) and *P_{rhaBAD}* (rhamnose-inducible) [120], showing stable and tunable expression with minimal leakiness across multiple cyanobacterial species, have been successfully established.

In summary, both native and synthetic inducible promoters have been explored in cyanobacteria. While native metal- and light-responsive promoters provide host compatibility, heterologous systems offer orthogonal regulation and better control after optimization. Ongoing exploration of cyanobacterial transcription factors and stress-responsive regulatory networks is expected to drive the development of more precise, tunable, and efficient inducible promoter systems for advanced gene regulation.

- **Constitutive promoters**

Constitutive promoters drive continuous gene expression independent of external stimuli and are essential tools for stable transgene expression in cyanobacteria. Several endogenous promoters, such as *P_{cpcB}*, *P_{psbA2}*, and *P_{rnpB}*, have been widely used for heterologous expression [145, 155, 168]. However, many native promoters are influenced by the circadian clock, resulting in variable expression across a 24-hour period [128, 169, 170]. This necessitates a focus on optimizing native promoters and developing synthetic variants to overcome such limitations. Truncation of native promoters has proven effective; for instance, *P_{cpc560}*, derived from *P_{cpcB}* of *Synechocystis* PCC 6803, achieved protein levels up to 15% of total soluble proteins [135, 137, 159], while a truncated *P_{psbA2S}* showed a fourfold increase in gene expression [71]. Similarly, a tandem hybrid promoter combining a truncated native PR from *Synechococcus* 7942 and the PS promoter from *E. coli* resulted in high expression efficiency [134].

High-throughput and bioinformatics-based approaches, including error-prone PCR and RNA-seq analysis, have enabled the generation of promoter libraries with a broad range of strengths [96, 148, 152]. Moreover, computational tools such as Virtual Footprint and bTSSfinder are useful in identifying new strong endogenous promoters for cyanobacterial systems [171].

3.4 Ribosome binding sites

RBSs are short nucleotide sequences located in the 5' untranslated region (UTR) of mRNAs that play a crucial role in initiating translation by recruiting ribosomes to the start codon. In polycistronic operons, each open reading frame (ORF) contains

its own RBS to ensure translation of all encoded proteins [87]. The canonical Shine–Dalgarno (SD) sequence (5'-GGAGG-3') pairs with the complementary anti-SD region of the 16S rRNA, positioning the start codon correctly within the ribosome [3, 87]. In cyanobacteria such as *Synechocystis* PCC 6803 and *Synechococcus elongatus* PCC 7942, both native and synthetic RBSs have been characterized and optimized for gene expression [87, 94]. For instance, the native 5' UTR of *Pcpc560* drives strong translation in *Synechocystis* [70], while synthetic sequences such as RBS* and BioBrick BBa_0034 provide high protein yields for fluorescent reporters [71]. Studies in *Synechocystis* and *S. elongatus* have revealed a broad range of RBS strengths, allowing modulation of gene expression at the translational level [6, 17, 71, 96, 139, 172, 173]. RBSs have also been used to coordinate multigene expression in synthetic operons or metabolic pathways. For example, in *S. elongatus* PCC 7942, differential RBS strengths were employed to balance expression of *alsS*, *alsD*, and *adh*, improving 2,3-butanediol production [174]. Similarly, optimization of RBS sequences enhanced ethylene (EFE) and limonene production in cyanobacteria [159, 175].

Computational design of RBSs has become an important strategy to fine-tune translation. Tools such as the RBS Calculator, UTR Designer, and RBS Designer use thermodynamic modeling to predict or design RBSs for desired translation rates [152, 176]. These tools show high predictive accuracy in *E. coli* ($R^2 > 0.8$) but perform less reliably in cyanobacteria, likely due to species-specific translational contexts [152, 172]. Despite the limitations, in silico RBS design combined with experimental validation has enabled substantial improvements in heterologous protein expression across cyanobacterial species such as *Anabaena* sp. PCC 7120, *Synechocystis* PCC 6803, and *S. elongatus* PCC 7942 [69, 71, 172]. Future research aims to develop cyanobacteria-specific RBS prediction tools to enhance accuracy and facilitate easier synthetic biology applications [139].

3.5 Riboswitches

Riboswitches are cis-acting regulatory RNA elements located mainly in the 5' UTRs of mRNAs that modulate gene expression by changing their structure upon binding to specific ligands such as metabolites, ions, or signaling molecules. Each riboswitch consists of two parts: an aptamer domain, which binds the ligand, and an expression platform, which mediates gene regulation by influencing transcription, translation, or mRNA stability [177]. To date, at least 56 classes of natural riboswitches have been identified, responding to compounds such as thiamine pyrophosphate, cobalamin, glycine, lysine, Mg^{2+} , Mn^{2+} , c-di-GMP, and ppGpp [178].

In cyanobacteria, functionally characterized native riboswitches include the glutamine-I riboswitch, which regulates glutamine synthetase activity in *Synechocystis* PCC 6803 [151], and cobalamin-dependent riboswitches in *Nostoc* sp. PCC 7120 [179] and *Synechococcus* PCC 7002 [180]. These native elements participate in nitrogen assimilation and vitamin B₁₂-related metabolism, but their limited ligand affinity and potential interference with essential metabolic pathways restrict their practical application [177, 181, 182]. To overcome these challenges, synthetic riboswitches have been developed to provide precise, tunable, and protein-factor-independent gene regulation [182]. Among them, the theophylline-dependent riboswitch, a modified RNA element that exposes or hides the RBS upon binding theophylline, has become the most widely used system in cyanobacteria [183–185]. Initially implemented in *Synechococcus* PCC 7942, it achieved up to 190-fold

induction with negligible basal expression [183] and was later successfully applied to *Synechocystis* PCC 6803, *Anabaena* PCC 7120, *Leptolyngbya* BL0902, and *Synechocystis* WHSyn [118, 154, 186]. A cobalamin-dependent riboswitch from *Synechococcus* PCC 73109 has also been adapted for *Synechococcus* 7002, offering low leakiness and about six-fold induction [180]. However, its applicability is limited to cobalamin-auxotrophic species, since others can synthesize the cofactor internally [187].

Overall, riboswitches provide compact, tunable, and ligand-responsive RNA tools for gene regulation in cyanobacteria. Their independence from protein inducers, modularity, and fast response make them promising for metabolic engineering [188, 189]. Future work should aim at combining in silico structural design with experimental tuning to expand the number of functional riboswitches and enhance their versatility across diverse cyanobacterial species [190].

3.6 Marker and marker-less selection system

In cyanobacterial genetic engineering, marker-based selection typically involves inserting a gene of interest alongside an antibiotic resistance gene to identify successful transformants, and recombinants are confirmed using PCR, sequencing, or Southern blotting [10, 17]. However, for multiple or stable modifications, marker-less selection systems are preferred as they avoid the accumulation of antibiotic markers and allow repeated genome editing [87]. These systems often use a two-step process combining a positive selection marker (antibiotic resistance) and a negative selection marker (lethal gene) such as *sacB*, which produces toxic compounds in the presence of sucrose. Only cells that lose the *sacB* gene survive on sucrose, enabling the recovery of unmarked mutants [191]. This counter-selection strategy has been successfully applied in several cyanobacterial strains, including *Synechocystis* [17, 84, 192–195].

Early marker less systems in cyanobacteria relied on mutation-based selection, such as the *rps12* system in *Synechococcus elongatus* 7942, which used a streptomycin-sensitive phenotype for double selection [196, 197]. Later, new counter-selection methods were developed, including those based on organic acid toxicity (*acsA*/acrylate system) in *Synechococcus* PCC 7002, nickel-inducible toxin expression (*mazF* system) in *Synechocystis* PCC 6803 [73], and *nptI*–*sacB* cassettes for single-vector deletions [198]. Some systems were found to be strain-specific, as *sacB* selection fails in marine cyanobacteria like *Synechococcus* PCC 7002 due to salt sensitivity [199, 200]. Broader methods, such as the *aas* knockout-based selection using free fatty acid tolerance, work across both *Synechocystis* PCC 6803 and *Synechococcus* PCC 7002 [201]. The most recent advancement in marker less genome editing uses CRISPR-based technologies, which create targeted DNA breaks without requiring counter-selection genes, thereby simplifying and accelerating the process [75, 76].

3.7 CRISPR-derived tools

A recent breakthrough in cyanobacterial genome engineering is the application of CRISPR-derived technologies. These approaches are generally classified into two main types: Genome editing and CRISPRi. Genome editing involves creating targeted double-strand breaks using a CRISPR-associated (Cas) endonuclease guided by a single-guide RNA, followed by homologous recombination to introduce new DNA sequences [202], while CRISPRi employs a catalytically inactive Cas protein, such as

dead Cas9 (dCas9), to repress gene expression without cutting the DNA. Furthermore, cyanobacteria themselves are an abundant natural reservoir of diverse and novel CRISPR-Cas systems [203–205].

CRISPR-Cas-based genome editing enables a range of genetic modifications in cyanobacteria, including gene knock-ins, deletions, and site-specific point mutations. A recent advancement involves RNA-guided transposition using a CRISPR-associated transposase, successfully demonstrated in *Nostoc sp. PCC 7120* with Golden Gate vectors [206]. The introduction of CRISPR/Cas9 technology significantly enhanced the efficiency of homologous recombination in cyanobacteria, as first shown in *Synechococcus elongatus* strains PCC 7942 and UTEX 2973 [207, 208]. However, excessive Cas9 expression was found to be toxic to cells, a limitation that can be mitigated by employing Cas12a (formerly Cpf1) [74]. Cas12a differs from Cas9 in several important ways: it recognizes an AT-rich PAM sequence (5'-TTN-3'), which better suits the AT-rich nature of many cyanobacterial genomes; it can process precursor crRNAs into mature crRNAs autonomously, without the need for host RNase; and it introduces staggered double-strand breaks, thereby improving recombination efficiency [209]. The effectiveness of Cas12a-mediated editing in cyanobacteria was demonstrated in *S. elongatus* UTEX 2973, where genetic variations between this fast-growing strain and the slower-growing *S. elongatus* PCC 7942 were systematically analyzed. In that study, the pUH24 plasmid was first eliminated from UTEX 2973, and each of the 39 chromosomal SNPs was individually converted to its corresponding allele in PCC 7942 using the Cas12a system, highlighting its precision and robustness [210]. The Cas12a-based CRISPR system has been adapted for cyanobacterial genome editing through the pSEVA plasmid series, which utilizes the broad-host-range RSF1010 origin [211]. This system has been successfully applied across various cyanobacterial species and strains, including *Synechocystis sp.*, *Synechococcus elongatus* PCC 7942, UTEX 2973, and 11081, *Anabaena (Nostoc) sp. PCC 7120*, and *Chroococcidiopsis sp. B13* [74, 76, 211, 212]. To mitigate Cas9-associated toxicity, another strategy involves regulating its expression using a theophylline-responsive riboswitch, which allows precise and conditional control of Cas9 activity. This approach has been proven effective for CRISPR/Cas9-mediated genome manipulations in *Synechocystis*, including the introduction of insertions and deletions of varying lengths [213]. This is extensively reviewed in the literature [75, 214].

CRISPRi uses catalytically inactive enzymes, such as dCas9 or dCas12a, which bind to DNA without cutting it, allowing for the repression of essential genes. The first demonstration of CRISPRi in cyanobacteria was in *Synechocystis sp. PCC 6803*, where a gene encoding green fluorescent protein (GFP) was successfully silenced [215], followed by the repression of essential genes like *rne*, encoding endoribonuclease E [75]. Since then, CRISPRi has been applied for metabolic engineering in several species, including *Synechocystis*, *S. elongatus* PCC 7942, *Synechococcus sp. PCC 7002*, and *Nostoc sp. PCC 7120* [9, 72, 153, 216].

Since CRISPRi causes partial repression rather than complete knockout, it enables genome-wide studies using single-guide RNA (sgRNA) libraries. For instance, an inducible CRISPRi library in *Synechocystis* targeting over 3,500 protein-coding genes and 1,800 noncoding RNA (ncRNA) loci was developed [217]. This approach allowed high-throughput gene fitness analysis under various growth conditions, helping identify key genes and enzymes with dual metabolic functions. The system was further used to improve L-lactate production, where repression of genes such as

gltA (citrate synthase) and *pcnB* (CCA-tRNA nucleotidyl transferase) enhanced yield [217]. Additional targets like *sdhB* and *ssr2016*, involved in redox balance and cyclic electron flow, also correlated with improved productivity under controlled light conditions.

Interestingly, CRISPRi screens also revealed that several ncRNAs influence fitness and metabolic regulation, highlighting their potential roles in fine-tuning cyanobacterial metabolism [218–221]. One such regulatory RNA, *ApcZ*, derived from the 3' UTR of the *apcC* gene, links light harvesting with energy dissipation by modulating orange carotenoid protein expression. These findings emphasize CRISPRi's potential not only for targeted gene repression but also for uncovering complex regulatory networks in cyanobacteria.

4. Applications of genetic engineering in cyanobacteria

Genetic engineering of cyanobacteria has opened up vast possibilities for sustainable biotechnological applications, driven by their ability to convert solar energy and atmospheric CO₂ into valuable bioproducts [159]. Engineered cyanobacteria have been explored as cell factories for the biosynthesis of renewable fuels, chemicals, and high-value proteins [159]. Through the integration of synthetic biology tools, metabolic pathways have been redesigned to enhance the production of compounds such as ethanol, hydrogen, isoprene, lactate, and 2,3-butanediol, positioning cyanobacteria as promising green biofactories [4–7, 159]. Recent advances have also extended to protein production, where cyanobacteria are engineered to express recombinant enzymes, pharmaceuticals, and nutraceuticals directly from atmospheric nitrogen and CO₂ [222]. Such systems provide a renewable alternative to conventional protein sources like soy or animal feed, reducing land and fertilizer dependency while minimizing greenhouse gas emissions [222]. Cyanobacterial strains have been optimized for industrial protein bioreactors, offering high yields under controlled photobioreactor conditions [104, 223]. Moreover, cyanobacteria are being exploited for the biosynthesis of bioactive metabolites, including pigments, carotenoids, polysaccharides, and antioxidants, with potential applications in the pharmaceutical, cosmetic, and nutraceutical industries [12, 224]. Genetic and metabolic engineering approaches have enhanced these pathways for improved yield and specificity [10, 11, 225]. Synthetic gene designs combining engineered promoters, RBSs, and terminators now enable fine control over protein expression and carbon flux, enhancing the production of amino acids and storage proteins [9, 159].

Together, these developments position cyanobacteria as a powerful platform for sustainable biotechnology. By combining metabolic engineering, CRISPR-based genome editing, and synthetic biology design principles, modern cyanobacterial systems can produce renewable biofuels, high-value chemicals, and nutritious proteins efficiently, while supporting carbon-neutral industrial processes [159, 222].

5. Future prospects

The progressive development of genetic tools in cyanobacteria reflects one of the most remarkable transitions in microbial biotechnology, from basic physiological

studies to precise genome engineering and pathway optimization. Each decade brought a critical advance: the establishment of transformation systems in the 1980s, the construction of shuttle vectors and selectable markers in the 1990s, genome sequencing in the late 1990s, and the emergence of synthetic biology and CRISPR/Cas technologies in the twenty-first century. Together, these milestones have transformed cyanobacteria into robust model organisms capable of supporting complex genetic designs. Modern toolkits now allow controlled expression of heterologous and synthetic pathways, marker-less genome modifications, and orthogonal regulatory systems compatible with photosynthetic metabolism. Meanwhile, the future of cyanobacterial genetic engineering is set to be revolutionized by the integration of artificial intelligence (AI), computational biology, and multiomics technologies, which together will redefine how cyanobacterial strains are designed and optimized. AI and machine learning (ML) models are increasingly being used to analyze large biological datasets and predict how DNA sequence variations influence gene expression and metabolic output [226, 227]. These algorithms can help identify strong promoters or RBSs, predict transcriptional efficiency, and design new synthetic regulatory elements tailored for cyanobacteria [228, 229]. Integrated within the Design–Build–Test–Learn (DBTL) cycle, AI-based models enable predictive design and refinement of genetic constructs, significantly reducing the time and cost associated with experimental optimization [227].

Multiomics-guided strain optimization, which integrates genomics, transcriptomics, proteomics, and metabolomics, has also become an important part of modern genetic engineering. These datasets feed into genome-scale metabolic models (GSMs) that simulate cellular metabolism and identify genetic targets for improving productivity [229]. For instance, AI-driven omics integration in *Synechocystis* sp. PCC 6803 enabled the reconstruction of regulatory networks that enhanced photosynthetic efficiency and redirected carbon flux towards desired metabolites [230]. Similarly, systems biology-based modeling in *Synechococcus elongatus* revealed metabolic responses to environmental stress, guiding the engineering of strains capable of producing higher yields of glycogen and fatty acids [231]. Thus, AI and multiomics integration can bridge the gap between computational predictions and experimental outcomes, making strain improvement more rational and data-driven.

Additionally, cell-free systems and minimal cyanobacterial chassis are emerging as powerful tools to overcome the inherent complexity of whole-cell systems. Cell-free systems are cellular extracts containing transcription and translation machinery, which allow precise and rapid testing of genetic constructs without the constraints of cellular regulation [232]. Minimal cyanobacterial chassis, such as *Synechococcus elongatus* UTEX 2973, are being developed as simplified yet highly efficient production hosts due to their fast growth and robust photosynthetic capacity [233]. These simplified systems make results more consistent, reduce unwanted cell activity, and make it easier to predict gene behavior.

Despite these innovations, the use of synthetic biology tools and strategies for designing and optimizing light-induced cyanobacterial cell factories is still at an early stage. Major challenges for future research include enhancing the efficiency of genetic tools, developing robust and versatile cyanobacterial chassis, simplifying and fine-tuning metabolic regulation, and advancing as well as integrating metabolic modeling approaches [223]. Additionally, scaling up genetic engineering for

industrial biotechnology is not easy, as engineered strains often exhibit instability, resource competition, and reduced productivity under variable outdoor conditions. Large-scale cultivation also faces limitations such as uneven light penetration, restricted CO₂ diffusion, and biofilm formation [230, 234]. Overcoming these barriers will require more research focused on integrating AI-driven predictive design, multiomics data, and cell-free prototyping with advanced bioprocess engineering and evolution-based strain improvement. These approaches will help in developing robust, scalable, and sustainable cyanobacterial biofactories capable of meeting the growing global demand for renewable fuels, feedstocks, and high-value biochemicals.

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Conflict of Interest

The authors declare no conflict of interest.

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Assessing an Algae-Based Biorefinery for Wastewater Treatment and Pigment Extraction

Hana Faulds, Jing Li and Hamse Kjerstadius

Abstract

Rising populations are placing significant strain on traditional waste management, prompting the need for circular systems in urban projects to recycle valuable nutrients from wastewater. Introducing a biorefinery framework, microalgae cultivation on digestate effluents produces pigments for natural dyes, reducing nutrient release and offering eco-friendly alternatives to synthetic products. Successful extraction of phycocyanin (the blue pigment) was achieved with an optimal 75% wastewater (WW) and 20% media composition, yielding the highest phycobiliprotein content (132.93 mg/g DW (dry weight)) coupled with impressive nutrient removal rates of 7.41, 1.31, and 11.73 (mg.L⁻¹.d⁻¹) for NH⁴⁺, PO₄³⁻, and TN (total nitrogen) respectively. The framework's scalability was evaluated in a Swedish urban development context, showcasing the potential of biorefinery concepts to convert waste into resources and supply natural pigments for textiles. Comparison with other studies showed that nutrient removal rates and PC yields are comparable to other wastewater-based systems with moderate biomass production, despite using a food-waste digestate stream. Our innovation also emphasized the high purity of phycocyanin (food-grade level), which many earlier studies don't report. Our study also provides insights into future development and discusses the limitations of practical applications.

Keywords: cyanobacteria, microalgae, natural dye, pigment, phycocyanin, spirulina, textiles, wastewater treatment

1. Introduction

For centuries, human consumption has followed a linear model, where resources are extracted, used only once, and discarded, typically relying on landfilling or incineration. The linear economy dominated after World War II until the concept of a circular economy gained traction in the late 2000s [1]. Wastewater has been increasingly seen as a resource, which provides added value derived from water, energy, nutrients, and materials [2, 3].

The concept of wastewater reuse, however, is not new. Ancient civilizations employed it for irrigation, fertilization, and disposal [4, 5]. Later, “sewage farms”

applied wastewater to extensive land areas for both disposal and agricultural purposes [6], but increasing populations exposed their public health and environmental risks. The contemporary system was built to direct wastewater away from its source, collecting large volumes close to the point of environmental disposal. Modern centralized wastewater treatment plants (WWTPs) improved sanitation by removing waste from urban centers, yet they remain logistically complex, energy-intensive, and limited in nutrient recovery [7]. Although today's centralized WWTPs aim to improve sanitation, they are not yet optimal in terms of circularity. Reclaimed water requires storage and redistribution, while biosolids from mixed waste often lack safe reuse potential.

To overcome these inefficiencies, decentralized systems are emerging. One such example is RecoLab, a pioneering facility in Helsingborg, Sweden, which separates waste streams at the household level into black water (from the toilet), gray water (from baths, showers, and laundry), and food waste (from kitchen waste grinders) [8]. This source-separation model enables more targeted treatment and recovery, generating biogas and clean fertilizers while reclaiming high-quality water, paving the way for a circular urban metabolism.

Building on this principle, the present study explores a novel biorefinery concept that leverages microalgae to simultaneously treat nutrient-rich wastewater and produce high-value natural pigments. These pigments have potential applications in textiles and as sustainable alternatives to synthetic dyes in inks, paints, and coatings. The experimental work is part of the *Green Services and Value-Creating Water* project, a collaboration between RecoLab, Helsingborg City, and Lund University [9].

Using algae to overcome environmental problems as phytoremediation is a safe method, as macro-/microalgae are among the primary microorganisms that possess significant biosorption capacity to deactivate hazardous chemicals [10]. There is great potential shown for microalgae to complement other bioremediation processes to explore more effective industrial wastewater treatment for sulfur and metal recovery, as discussed in the role of sulfate-reducing bacteria (SRB) in the bioremediation of sulfur-rich wastewater [11]. While nutrient removal through microalgal treatment and the commercial use of algal pigments have been studied independently, a few approaches integrate both in a closed-loop biorefinery concept [12–14].

This study addresses that gap, focusing on the coupling of wastewater treatment with the coproduction of commercially relevant biochemicals, with specific attention to textile dye replacement [15]. Unlike earlier phases of the project, where mixed wastewater was used, this experiment employed food waste residuals, considered less contaminated and nutritionally sufficient for algal growth.

1.1 RecoLab treatment process

The black water and food waste streams have gained particular interest due to their elevated nutrient content. Food waste undergoes pasteurization at 70°C for one hour and then enters a digester, where anaerobic digestion produces biogas. The resulting digestates are nutrient-rich, especially in macronutrients like nitrogen and phosphorus. These elements are harnessed through struvite precipitation and nitrate extraction to produce biofertilizer from the liquid phase of the digestate. While this method effectively removes and recycles nutrients from wastewater,

concerns over excessive fertilizer usage and nitrogen run-off, contributing to eutrophication, have prompted a reevaluation of EU regulations. This includes revisions under the EC Nitrates Directive (91/676/EEC) and nitrate vulnerable zone (NVZ) legislation. Restrictions on the amount of nitrogen that can be reintroduced to the land have been implemented, with similar restrictions on phosphate fertilizers currently being introduced [16].

1.2 Microalgae biorefinery concept

Recently, evoking global interest lies in an alternative valorization pathway: the cultivation of commercially valuable microalgae biomass using digestate effluents. The concept of using microalgae for nutrient remediation was initially put forth over 60 years ago by Oswald and Gotaas [17], pioneers in this field. Significant progress has been made afterward, with recent findings indicating between 85% and 100% removal efficiency by different microalgae species [18]. The microalgal system for removing nutrients has shown high removal efficiencies of up to 40 mg N L⁻¹ d⁻¹ and 4 mg P L⁻¹ d⁻¹ for nitrogen and phosphorus, respectively, with the added benefits of not requiring an organic carbon source and generating no sludge [19]. Despite microalgae's efficiency in removing both inorganic and organic contaminants, their use in wastewater treatment is still limited. In cases where they are implemented, it is often only a polishing step, and there is little emphasis on using the biomass produced for valuable products [20]. By coupling the process of waste management with microalgae production, this model not only addresses the challenges of nutrient remediation but also offers a solution to the high production costs associated with microalgae. This is achieved by replacing the use of expensive media and nutrient solutions with the digestate effluent stream. As the economic potential of microalgal biomass becomes perceivable, its utilization in wastewater treatment is likely to increase.

1.3 Valorization of microalgal biomass

Microalgae, versatile organisms rich in protein, vitamins, essential oils, antioxidants, pigments, and other valuable metabolites, hold significant biotechnological and environmental importance [21]. The main commercial applications for microalgae biomass include animal feed, nutraceuticals, cosmetics, and human nutrition. While much research on the biorefinery concept of microalgae grown on wastewater emphasizes the application of the resulting biomass as animal and fish feed, there is a growing interest in the extraction of pigments from microalgal biomass for use as natural dyes or ink solutions to replace petroleum-based products that dominate the market [22]. This study initiates a lab-scale experiment for growing algae on digestate effluents to extract valuable pigments.

1.4 Study objectives

This research aims to:

1. Evaluate the capacity of microalgae to remove nitrogen and phosphorus from synthetic wastewater.

2. Assess the quality and yield of natural pigments extracted from the cultivated biomass.
3. Examine the technical feasibility and circular economic potential of using this biomass in pigment-based applications.

1.5 Hypothesis

Microalgae cultivated in nutrient-rich wastewater can efficiently remove key pollutants while producing natural pigments suitable for use in the textile industry. This integrated process offers a scalable, decentralized solution for advancing circularity in wastewater management and sustainable production systems.

2. Materials and methods

This study comprised two main phases. Firstly, an initial growth experiment was conducted to test the photobioreactor system, assess the growth rate, and determine suitable media composition, followed by a main cultivation phase. All materials and methods refer to the master thesis project [23].

2.1 Materials

*2.1.1 *Arthrospira platensis* (A. platensis) stock culture and inoculum preparation*

Arthrospira platensis (A. platensis) starting culture was supplied by Health Algae. Upon its arrival, the 250 mL culture was placed under a white-fluorescent light to allow the culture to recover from any degradation during shipping. On the following day, the culture was divided into two 2 L Erlenmeyer flasks, each containing 1 L of Zarrouk's medium, and placed in a climate chamber (Pol-EKO Aparatura)[24] under a temperature of 25°C, a photoperiod of a 14:10 hours light: dark cycle, and a light intensity of 100%. On the third day, aeration tubes with gas stones were introduced for stirring, receiving 5,000 mL of air per minute from an aquarium air pump (IRENUO Q7 HQ-602). These cultures were maintained under the specified conditions, with 10% of the culture replaced with fresh media every 2–3 days until inoculation.

2.1.2 Media formulation

In this study, the Zarrouk medium used was modified based on the method outlined by Baker et al., 2021. It comprises three distinct solutions and was not autoclaved after mixing to avoid the precipitation of metals. Solutions 1 and 2 were autoclaved separately, and Solution 3 was sterilely filtered. After these steps, all three solutions were combined. The composition of the medium is as follows: 500 mL/L Solution 1 (16.8 g NaHCO₃, 0.5 g K₂HPO₄ [per L]), 500 mL/L Solution 2 (2.5 g NaNO₃, 1 g K₂SO₄, 1 g NaCl, 0.2 g MgSO₄ · 7H₂O, 0.04 g CaCl₂, 0.01 g FeSO₄ · 7H₂O, 0.08 g NaEDTA [per L]), 1 mL/L Solution 3 (2.86 g H₃BO₃, 1.81 g MnCl₂ · 4H₂O, 0.222 g ZnSO₄ · 7H₂O, 0.079 g CuSO₄ · 4H₂O, 0.015 g MoO₃ [per L]).

Wastewater samples were collected from the digested influent of the food-waste stream (which was kept separate from black and gray water streams) at the wastewater treatment facility, RecoLab. Upon its arrival at the treatment facility, food waste undergoes pasteurization at 70°C for one hour to eliminate any present pathogens, and then it enters the anaerobic digester (AD), where it is maintained at 37°C for several days to facilitate sedimentation. The liquid phase settled above the sediment was collected for use in this study. These collected samples were immediately autoclaved at 120°C for 30 minutes in accordance with lab safety protocols to eliminate any remaining microorganisms. Following autoclaving, the wastewater underwent filtration using standard Whatman filter paper to remove larger suspended particles, enhancing the liquid's transparency for improved light exposure. The samples were then stored at 4°C until use to prevent any biological activity and nutrient degradation [23].

Zarrouk medium and wastewater mixtures: different compositions of wastewater to medium were tested in duplicates: 100% FWW, 75% FWW + 25% Zarrouk, and 50% FWW + 50% Zarrouk, to compare the growth dynamics of *A. platensis* on increasing concentrations of wastewater. Phase I had a total working volume of 600 mL, and Phase II was scaled up to 4 L each [23].

2.1.3 Equipment and photobioreactor set-up

For both the growth experiment and the scale-up phase, a makeshift photobioreactor system was established. In the growth experiment, 1 L glass laboratory bottles (DWK Life Sciences DURAN) were arranged in sequence, featuring inlet and outlet tubing to facilitate aeration and air transfer. To minimize the risk of contamination, both the inlet and outlet were equipped with 0.22 µm filters. Each glass bottle was positioned equidistantly and sequentially from the light source, ensuring uniform light exposure without any shadowing effects between duplicates. During the scale-up phase, 5 L glass bottles were employed, maintaining the same lighting, tubing, and temperature systems [23].

2.2 Methods: experimental design

The first step of the experiment aimed to examine *A. platensis* cultivation using three different wastewater-based growth media: (1) 100% FWW, (2) 75% FWW supplemented with 25% Zarrouk medium, and (3) 50% FWW supplemented with 50% Zarrouk medium. The cultures, with a combined working volume of 600 mL, were incubated for 21 days under conditions identical to those used for the inoculum cultures. This experiment resulted in identifying the optimum ratio of wastewater to media. In the second step of the experiment, a composition of 75% FWW and 25% Zarrouk medium was exclusively used. The working volume for this experiment was scaled up to 4 L, and nutrient removal was monitored at increased intervals throughout the cultivation period. In both steps, the pH was adjusted to 9 using a 5 N NaOH solution, and it was maintained within the range of 9–11 during cultivation by using 5 N HCl or 5 N NaOH solutions. The light intensity was consistently set to 100%, following the climate chamber settings provided by white-fluorescent lights. The temperature was maintained at a constant $25 \pm 3^\circ\text{C}$ for the first experiment and at $28 \pm 3^\circ\text{C}$ for the second; however, humidity was not controlled. All experiments were performed in duplicate under nonaseptic conditions, with

continuous aeration provided by an air pump at a rate of 5 L air/min. The experimental design and climate chamber conditions are visually outlined in **Figure 1**. Replicates of samples were used for experiments [23].

2.3 Analysis

2.3.1 pH and conductivity

During both experiments, pH and conductivity were monitored using the HANNA Combo® HI98129 instrument. This instrument was also used to check the pH of wastewater samples and other growth media used.

2.3.2 Dry weight, optical density, and standard curve

To facilitate sampling, a calibration curve was created to examine the relationship between dry weight and optical density. A stock culture of *A. platensis* and five serial dilutions, each with a 20 mL volume, were prepared. The optical density for each dilution was then measured using a spectrophotometer (Ultrospec 2100pro, Amersham Biosciences) at a wavelength of 565 nm. Dry weight determination followed a standard protocol: 0.45 µm membrane filters were microwaved for 4 minutes, placed in a desiccator overnight, and their initial weight was recorded. Afterward, 10 mL of each dilution was filtered, washed three times with 5* × 10 mL of cold distilled water to collect the algal cells, microwaved for 8 minutes, and left to dry in the desiccator overnight. The final weight of the filters was recorded, and the dry weight was calculated using the equation below [23].

$$\text{Dryweight}(\text{mg/mL}) = \frac{\text{initialweightoffilter} - \text{finalweightoffilter}}{\text{volumefiltered}(\text{mL})} \quad (1)$$

A standard curve was plotted in **Figure 2** for the data within the range of 0–1.4 g/L dry weight of culture, and a linear regression was performed to obtain the linear equation and the R-squared value of the curve.

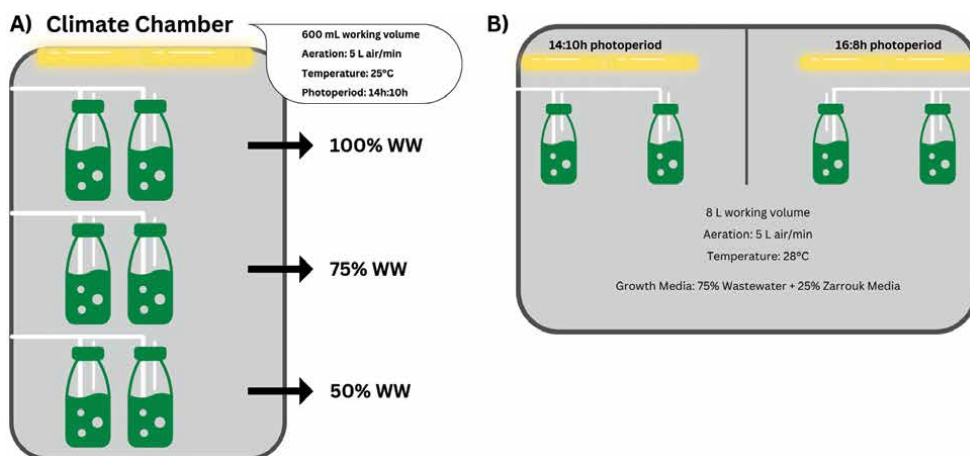


Figure 1.

Graphic layout and parameter specifications for (A) optimization experiment 1 and (B) scale-up experiment 2.

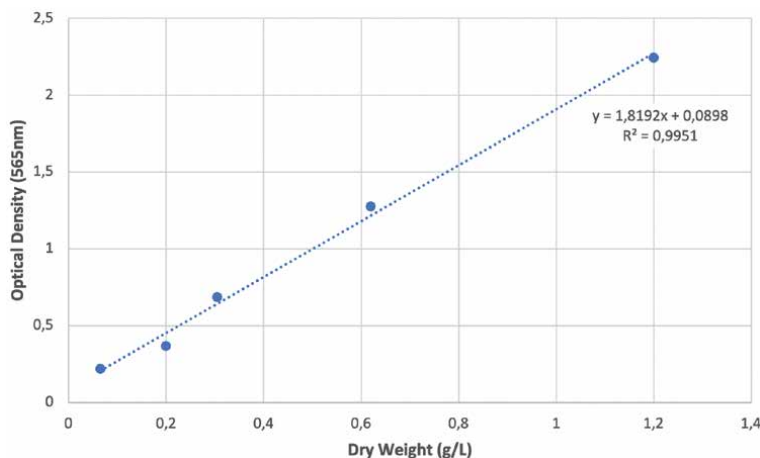


Figure 2.

Linear correlation between optical density at 565 nm and dry weight measurements (g/L).

2.3.3 Phycobiliprotein content

Phycobiliproteins (PBPs) are a special class of water-soluble pigment–proteins found in cyanobacteria (blue-green algae), red algae, cryptophytes, and some other microalgae. There are several types of PBPs, which are named for their absorption color:

- Phycocyanin (PC): Blue pigment
- Allophycocyanin (APC): Bluish pigment
- Phycoerythrin (PE): Red pigment
- Phycoerythrocyanin (PEC): bluish-red (pink-orange)

The quantification of PBPs in the biomass produced during both experiments followed similar methods outlined by Arashiro et al. [25], with relevant adaptations. Briefly, 5 mL samples, taken every 2–4 days, were immediately centrifuged at 1,160 g for 15 minutes, washed twice with cold distilled water, and the resulting biomass pellet was frozen at – 21°C for further quantification. The pellets were then resuspended in 4 mL of concentrated 37% HCl and kept in the dark at 4°C for 24 hours. Optical densities were then measured at 280 nm, 562 nm, 615 nm, 652 nm, and 620 nm. The PC, APC, and PE concentrations and yield were calculated using the equations proposed by [26] as listed below.

$$\text{PC (mg/mL)} = [A_{615} - (0.474 * A_{652})] / 5.34 \quad (2)$$

$$\text{APC (mg/mL)} = [A_{652} - (0.208 * A_{615})] / 5.09 \quad (3)$$

$$\text{PE (mg/mL)} = [A_{562} - (2.41 * \text{PC}) - (0.849 * \text{APC})] / 9.62 \quad (4)$$

Purity of PC was determined according to the ratio A620/A280, as defined by Cuellar-Bermudez et al. (2015), where A615, A652, A280, A620, and A562 correspond to the absorbance measured at the respective wavelengths [27]. The purity of PC is related to the total amount of protein present in the extraction product [28].

2.3.4 Nutrient removal

Nutrient removal was measured by monitoring concentrations of ammonium (NH₄-N), phosphorus (PO₄³⁻-P), total nitrogen (TN), and total organic carbon (TOC) using the following Hach test kits: LCK303 ammonium (2–47 mg/L), LCK302 ammonium (47–130 mg/L), LCK350 phosphorus Total (2–20 mg/L), LCK348 phosphorus total (0.5–5 mg/L), LCK438 LATON TN (100–250 mg/L), and LCK386 Total Organic Carbon (30–300 mg/L), following the test protocols provided using a portable spectrophotometer (Hach DR3900).

2.3.5 Calculations

For simplicity, the specific growth rate (μ) of *A. platensis* throughout this project was estimated using the equation below, where X is cell concentration (g/L) and t is time (days).

$$X(t) = X_0 * e^{\mu t} \quad (5)$$

Biomass productivity (mg · L⁻¹ · d⁻¹) was determined by Eq. 6, where X_{max} (g · L⁻¹) was the maximal biomass concentration, X_0 (g · L⁻¹) was the initial biomass concentration, and T_{max} was the time for biomass to reach maximum (days).

$$Biomassproductivity(mg.L^{-1}.d^{-1}) = \left[X_{max} - \frac{X_0}{T_{max}} \right] * 1000 \quad (6)$$

The removal efficiency (%) of pollutants from wastewater was evaluated by Eq. 7, where C_{in} (mg · L⁻¹) was the initial concentration of the pollutant (NH₄ PO₄³⁻, and TN), and C_{out} (mg · L⁻¹) was the concentration of the residual pollutant when the biomass concentration reached its maximum.

$$Removalefficiency(\%) = \frac{C_{in} - C_{out}}{C_{in}} * 100 \quad (7)$$

The average removal rate (mg · L⁻¹ · d⁻¹) of each pollutant was calculated by Eq. 8, where T_{max} was the time (days) for biomass to reach its maximum.

$$Av.Removalrate(mg.L^{-1}.d^{-1}) = \frac{C_{in} - C_{out}}{T_{max}} \quad (8)$$

2.3.6 Statistical analysis

All assays were performed in duplicates, and statistical analysis was conducted using JASP (JASP-stats, Amsterdam, Netherlands). The average values measured during both experiments were analyzed using multifactor analysis of variance (ANOVA), and the significant differences among growth media variations and PC production were determined using a two-tailed T-test at a 95% confidence interval.

3. Results

3.1 Growth analysis

The initial growth experiment of *A. platensis* involved different concentrations of digestate effluent, as shown in **Figure 3A**. The results for cultivation grown in 100% WW indicate minimal growth. On the contrary, cultivations on 75% and 50% WW exhibited notable biomass concentrations, reaching 1.6 g L⁻¹ after 17 days and 2.09 g L⁻¹ after 18 days, respectively.

The absence of growth with 100% WW indicates that wastewater alone may lack essential elements such as alkalinity, salinity, and additional nutrient components like potassium and trace elements, which are necessary for the optimal growth and health of *A. platensis*. Potential factors, such as ammonium-inhibition or the influence of specific bacterial populations, call for more in-depth investigation. Given that the objective was to establish growth primarily on WW while ensuring

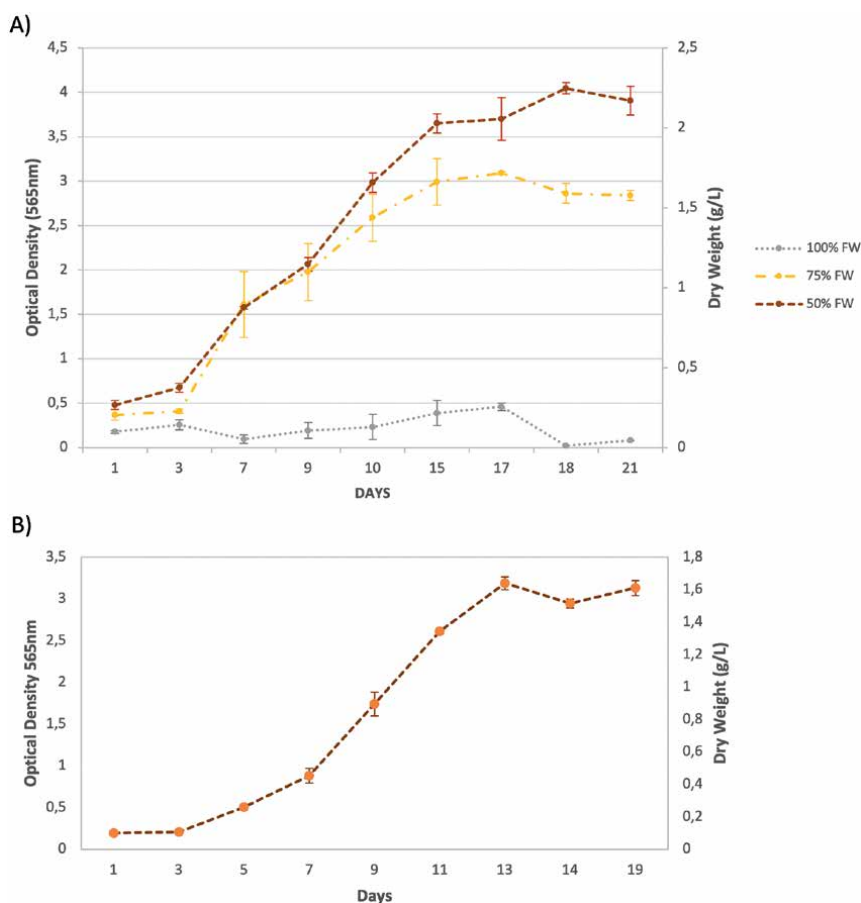


Figure 3. Biomass growth of *A. platensis* during (A) the optimization experiment (working volume: 600 mL) on growth media containing different amounts of WW (100%, 75%, 50%) supplemented with Zarrouk media, and (B) main cultivation (working volume: 4 L) in 75% WW, measured as optical density at 565 nm and converted to dry weight (g/L).

substantial biomass growth, the growth rate measured in the 75% WW cohort was considered sufficient and was thus selected as the optimal medium composition for the main cultivation, despite the higher biomass concentration observed in the 50% WW cohort. During the main cultivation, the total volume increased from 600 mL to 4 L, achieving a peak biomass concentration of $1.64 \text{ g} \cdot \text{L}^{-1}$ at a growth rate of $0.34 \text{ g} \cdot \text{d}^{-1}$ after only 13 days, as shown in **Figure 3B** [23].

3.2 Nutrient removal

Ammonium (NH_4^+), orthophosphate (PO_4^{3-}) and TN, illustrated in **Figure 4**, functioned as nutrient markers. Their concentrations were monitored at specific time points during the cultivation of *A. platensis* to evaluate the efficiency and rate of nutrient removal.

Results in **Figure 4** show that the removal rate and efficiency were impressive for all three nutrient markers, averaging 7.41 , 1.31 , and $11.73 \text{ (mg} \cdot \text{L}^{-1} \cdot \text{d}^{-1})$ for NH_4^+ , PO_4^{3-} , and TN, respectively. The initial concentrations of nutrients (NH_4^+ , PO_4^{3-} , and TN) measured before *A. platensis* inoculation were 183.55 , 20.6 , and 96.6 (mg/L) , respectively. **Figure 3** also demonstrates that, in every instance, concentrations fell below the accurate detection range by day 10 of cultivation, and both nitrogen and phosphorus sources were approaching depletion by day 14. This rapid nutrient removal aligns with the observed lack of biomass growth after day 14, indicating that the cultivations entered a stationary phase. Efficiency of removal (%) was calculated at 96%, 95%, and 88% [23].

3.3 Phycocyanin concentration and purity

The PBP content of the biomass was examined during both growth analyses, as it is recognized to have a positive correlation with cyanobacterial biomass concentration [29]. **Figure 5** illustrates the concentration and purity of PC extracted from the final biomass. Concentration indicates the amount of PC per gram of dry

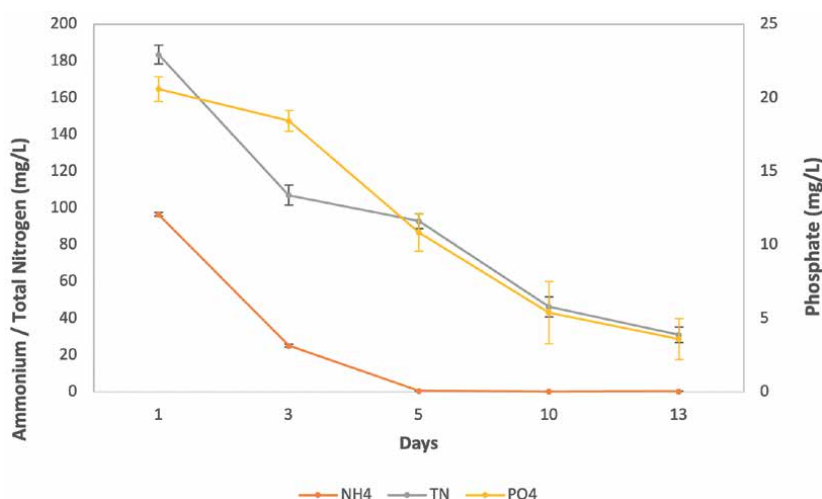


Figure 4.

Nutrient removal during main cultivation (working Vol. 4 L) as shown by ammonium (NH_4^+), total nitrogen (TN), and phosphate (PO_4^{3-}) consumption by *A. platensis* throughout cultivation.

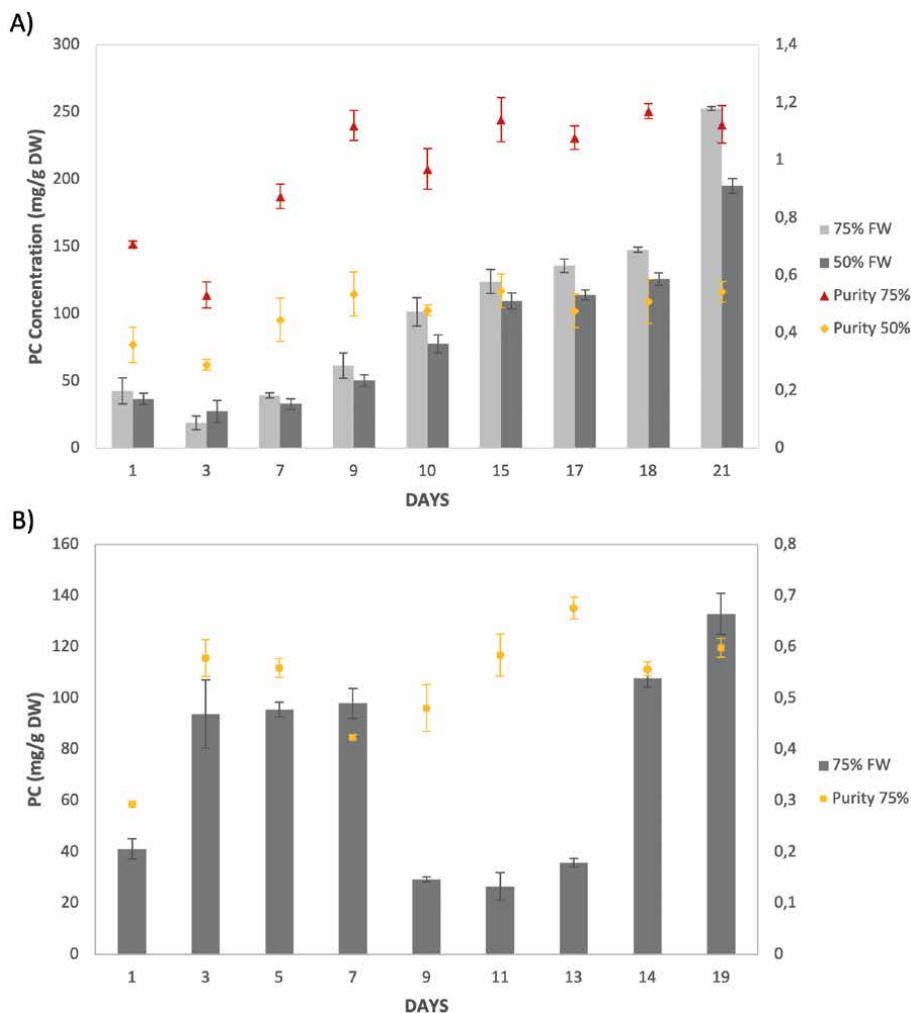


Figure 5. Phycocyanin content in total biomass and respective purity during (A) Phase I of *A. platensis* cultivation at 600 mL working volume with different concentrations of WW and (B) Phase II at a working volume of 4 L with 75% WW concentration in the growth media [23].

weight, while purity defines the relationship between the presence of PC and other contaminating proteins in the extracted sample [30].

The purity ratio, determined by the A_{620}/A_{280} ratio, indicates high purity when values are above or equal to 0.7, signifying food-grade pigment, and values above or equal to 4.0 correspond to reagent and analytical-grade pigment [31]. In the initial growth analysis (**Figure 4B**), there was a progressive increase in PC content measured throughout cultivation, reaching final concentrations of 252.76 mg/g DW and 195.2 mg/g DW for the 50% and 75% WW cultivations, respectively. Previous studies have observed similar patterns in the influence of wastewater-based media composition on pigment concentration, with higher amounts of NaHCO_3 and NaCl resulting in the maximum PC concentration [29]. Arashiro et al. (2020) also reported similar findings, achieving maximum PC concentrations (approx. 200 mg/g DW)

with a combination of standard growth medium and wastewater [14]. Factors such as nitrogen concentration, bacterial populations, salinity, and pH can impact PBP concentration during cultivation, suggesting a need for further analysis of these variables. In summary, the optimal conditions for PBP production were found with a media composition of 75% wastewater and 25% Zarrouk media [23].

3.4 Assessing scale-up potential

Although the initial lab-scale testing was promising, scaling up any algae cultivation plant presents challenges and requires a comprehensive commercial assessment. However, a full economic feasibility study was beyond this project's scope. Typically, production costs decrease with increased capacity. However, the proposed model is constrained by the volume of liquid effluent leaving the anaerobic digester (AD), determined by the total incoming volume from residential usage. Due to innovative technology and water-saving ambitions in the Oceanhamnen development, an average of only 5 L per person per day of wastewater enters the facility, compared to 250 L per person per day at a traditional WWTP. Approximately 4.5 L is estimated to leave the AD as liquid effluent, utilized as microalgae growth media. Considering these volumes and assuming a total initial population of 1,000 residents, as a conservative estimation, the process feasibility was assessed with an extraction capacity between 5,625 and 6,000 L/day. Assuming a constant growth rate (0.34 d^{-1}) and maximum biomass concentration (1.64 g/L) from lab-scale growth analysis, the estimated annual biomass production is 2,398.5–2,558.4 kg, equating to a minimum annual extraction of 191.88–204.6 kg (8% of total biomass) C-phyococyanin. To consider this process for commercial application, the system would need to have an annual extraction of closer to one ton, around 10 times the current estimated extraction capacity. The mass balance for nitrogen and phosphate over the proposed cultivation system has been outlined in **Figure 6** below, illustrating the potential use of microalgae as a nutrient extraction step during wastewater treatment, where most of the nitrogen and phosphate are removed from the water stream by the resulting biomass [23].

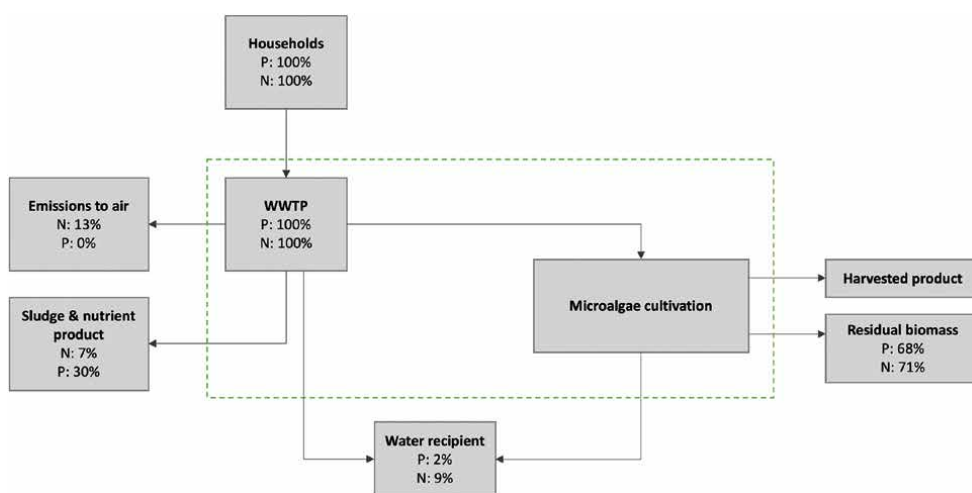


Figure 6. Mass balance for nitrogen (N) and phosphate (P) over the proposed cultivation system[23].

4. Discussion

This study has demonstrated the dual potential of *A. platensis* cultivation on food-waste-derived wastewater, achieving high nutrient removal efficiencies while producing a commercially relevant pigment: PC.

4.1 Growth dynamics

The observed stationary phase around day 14 suggests that, beyond nutrient depletion, additional factors may have limited productivity. Possible explanations include light penetration constraints in denser cultures, pH drift due to metabolic activity, and accumulation of inhibitory metabolites. Similar growth slowdowns in wastewater-based media were reported by Arashiro et al. [14] and Papadopoulos et al. [29], supporting the need for optimized photoperiods, mixing, and CO₂ supplementation.

4.2 Nutrient removal

Our nutrient removal results align with previous studies [14]. Their reported values, ranging from 52% to 100% for TN and between 73% and 82% for phosphate removal during cultivation on industrial wastewater, showed no significant effect from varied light conditions. The particularly high ammonium removal rate ($7.41 \text{ mg} \cdot \text{L}^{-1} \cdot \text{d}^{-1}$) indicates that digestate-derived wastewater provides a suitable nitrogen source, although ammonium toxicity may partly explain poor growth in 100% WW treatments. This reinforces the importance of media balancing when targeting both remediation and biomass productivity.

Observing the removal of COD, heavy metals, and additional forms of nitrogen throughout cultivation could offer a more comprehensive evaluation of this strain's capability to treat the wastewater stream. This aspect is recommended for future research.

4.3 Pigment production

PC yields (195–253 mg/g DW) are comparable to those reported in mixed wastewater-media systems [14] [29], as shown in **Table 1**. Importantly, the purity values achieved ($A_{620}/A_{280} \geq 0.7$) reached food-grade standards, indicating strong potential for textile and nutraceutical applications. The method employed here, while effective, could benefit from downstream process optimization to increase recovery rates and reduce costs, as highlighted by Borowitzka [31].

4.4 Comparison with previous studies

The nutrient removal rates and PC yields are comparable to other wastewater-based systems, despite using a food-waste digestate stream (novel source); see **Table 1**.

Our innovation emphasized the high purity of PC (food-grade level), which many earlier studies don't report. It is also noted that the biomass productivity is moderate compared to some, but still promising given decentralized urban wastewater volumes.

Study	Microalgae species	Wastewater type and medium	Biomass productivity (g·L ⁻¹)	N removal (%)	P removal (%)	Phycocyanin yield (mg/g DW)	Notes
This study (2025)	<i>Arthrospira platensis</i>	75% food-waste digestate + 25% Zarrouk	1.64	96% (NH ₄ ⁺), 88% (TN)	95%	195–253	High purity (≥0.7 A620/A280); poor growth in 100% WW
Cañizares-Villanueva et al. [18]	Cyanobacteria	Diluted swine wastewater	1.2–1.5	~80–90%	~70%	~180	Aerated systems: strong dependency on dilution ratio
Zhu [19]	Mixed microalgae	Synthetic wastewater	1.0–2.0	85–100%	80–90%	n/a	Review – emphasizes the need for nutrient balancing
Arashiro et al. [14]	Cyanobacteria	Industrial wastewater + growth medium	1.8–2.2	52–100%	73–82%	~200	Comparable pigment yields; light not limited.
Papadopoulos et al. [29]	<i>Arthrospira platensis</i>	Brewery wastewater	1.5–1.8	~90%	~80%	~220	Higher PC with elevated NaHCO ₃ and NaCl.
Borowitzka [31]	Review (various)	Various	0.8–2.5	70–95%	60–85%	100–400	Notes commercial potential but stresses cost barriers

Table 1.
Comparison with other similar studies.

4.5 Scale-up potential

Borowitzka [31] briefly reviews the main existing and potential high-value products that can be derived from microalgae and considers their commercial development, with a particular focus on the various aspects that need to be addressed on the path to commercialization, using the experience gained in the commercialization of existing algae products. While our mass balance indicates feasibility at the pilot scale, the production volume estimated (~200 kg C-phyococyanin/year) is well below industrial demand thresholds (>1 ton/year). Similar economic challenges have been noted in other microalgae biorefineries [19, 31] for diverse applications. Scaling may require integration with multiple urban digesters, improved photobioreactor efficiency, or coupling with additional high-value coproducts (e.g., antioxidants, bioactive peptides) to strengthen economic viability. When integrating wastewater treatment processes for sustainable energy recovery, microalgae have shown impressive energy output potential, with algal biomass having an energy potential of 60–160 MWh per hectare per year, depending on the seasonality and treatment method [32]. Resource recovery overall is still limited in practice, as noted in a critical review, which states that despite many technical options, “only a few technologies have been implemented and a shift from wastewater treatment plants towards water resource recovery facilities still seems far away” [33]. Future endeavors need to be undertaken to advance the role of microalgae in resource recovery further.

4.6 Future work

This study focused on nutrient removal and pigment extraction but did not assess COD, heavy metals, or microbiome shifts during cultivation. Future work should include:

- Expanded monitoring of additional pollutants (COD, metals)
- Investigation of microbial community dynamics and their role in productivity
- Optimization of light intensity, photoperiod, and CO₂ aeration
- A techno-economic assessment of scale-up under decentralized wastewater treatment models

To investigate algae's role in wastewater treatment, there is huge potential that can be further explored for a circular future; for example, soil enzymes from microalgae have great potential for their application in biotechnology and agriculture [34].

5. Conclusion(s)

This paper presents a biorefinery framework for cultivating microalgae using digestate effluent and extracting valuable pigments from the biomass. Successful growth of *A. platensis* on municipal waste streams confirms its potential. While

wastewater alone didn't sustain growth, 75% and 50% wastewater cultivations showed sufficient biomass growth. The optimal 75% wastewater and 20% media composition produced the highest PBP content. Impressive nutrient removal rates were observed for nitrogen (N) and phosphorus (P) in all cultivations, indicating nutrient recovery efficiency. We assessed scalability for the entire Oceanhamnen development, a step toward circular community development.

Despite these achievements, further optimization is needed to improve growth rate and biomass productivity, exploring factors like photoperiod, light intensity, and CO₂ levels. Comprehensive evaluation, including COD, heavy metals, and additional nitrogen forms removal, is essential. Future work should address the bacterial community's impact on biomass productivity and PC concentration and purity. This framework holds promise for the textile industry, paints, inks, and natural dye products but requires ongoing refinement for broader applications and enhanced efficiency. Microalgae, as a resource recovery medium in general, is a great candidate to be further investigated.

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Conflict of interest

The authors declare no conflict of interest.

Notes/thanks/other declarations

Place any other declarations, such as “Notes”, “Thanks”, etc., before the References section. Assign the appropriate heading.

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Limnospira platensis LEGE 231238: Exploring Commercial Potential through Comprehensive Biochemical and Taxonomic Analysis

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Abstract

The strain *Limnospira platensis* LEGE 231238, isolated from the saline Alalay Lake in Bolivia, was identified through a polyphasic approach. The biochemical characterization revealed that the dry biomass presented (% of dry weight) protein (45.40 ± 0.54), carbohydrates (27.84 ± 0.25), ash (19.90 ± 0.32), lipids (6.86 ± 0.18), and C:N ratio (5.54 ± 0.17). Lipid extracts revealed $46.23 \pm 0.60\%$ polyunsaturated fatty acids (FA), $41.24 \pm 0.72\%$ saturated FA, with palmitic acid (FA 16:0) accounting for $39.05 \pm 0.66\%$ and γ -linolenic acid (FA 18:3 *n*-6) for $31.32 \pm 0.30\%$. Nutritional quality indices included atherogenic ($0.75 \pm 0.03\%$), thrombogenic ($1.32 \pm 0.04\%$), and hypocholesterolemic/hypercholesterolemic index ($0.45 \pm 0.03\%$). Acetonic extracts contained 78.66% chlorophyll-*a*, 16.18% total carotenes, and 5.11% xanthophylls. The aqueous extract showed the following contents ($\mu\text{g mg}^{-1}$): phenolics with 15.64 ± 1.76 as gallic acid equivalents, sugars with 7.19 ± 0.75 of glucose equivalents, chlorophyll-*a* with $5.84 \pm 1.87 \text{ mg g}^{-1}$ of dry extract, and total chlorophylls with $10.02 \pm 2.06 \text{ mg g}^{-1}$ of dry extract. Phycobiliprotein content revealed concentrations of phycocyanin, allophycocyanin, and phycoerythrin at 84.88 ± 8.29 , 53.11 ± 6.21 , and $20.25 \pm 3.12 \text{ }\mu\text{g mg}^{-1}$ of dry extract, respectively. The nutritional profile of LEGE 231238 is comparable to that of other *Spirulina* strains currently available on the market, supporting its suitability as a food supplement. This work represents the first published report of an *L. platensis* isolate from Bolivia and underscores its strong potential for commercial exploitation.

Keywords: fatty acids, Lege-CC, nutrition, phylogeny, pigments, phenols, phycobiliproteins, protein, *Spirulina*, sugar

1. Introduction

Cyanobacteria, previously referred to as blue-green algae, are among the oldest oxygenic photosynthetic organisms on Earth and play a crucial role as primary producers with a global distribution [1]. Their high biotechnological potential has been widely documented, largely attributed to their rapid growth rate, ability to synthesize pigments such as chlorophyll and phycocyanin (PC), and capacity to accumulate diverse bioactive compounds under variable environmental conditions [2, 3]. These metabolites exhibit diverse applications in human health, encompassing cardioprotective, anti-inflammatory, and antioxidant effects [4–6].

In human nutrition, species of the genus *Limnospira* Nowicka-Krawczyk, Mühlsteinová and Hauer (commercially known as *Spirulina*) deserve special attention. They are an exceptionally rich source of protein, representing 70% of dry weight (DW), considerably higher than conventional sources such as meat, which contains around 22% [7–9]. Moreover, *Limnospira* is a source of polyunsaturated FA (PUFAs), particularly gamma-linolenic acid, along with a wide range of vitamins (A, D, E, K, C, and B-complex) and micronutrients such as calcium, iron, magnesium, potassium, and selenium. It is also rich in bioactive compounds, including PC, known for its strong antioxidant activity, and several carotenoids, including β -carotene, canthaxanthin, lutein, astaxanthin, and zeaxanthin, contributing to its nutraceutical value. Its cell wall contains polysaccharides like peptidoglycans and hemicellulose, potentially acting as prebiotics with immunomodulatory properties [10–13].

This nutritional value is historically known and can be traced back to the sixteenth century, when the Aztecs harvested *Limnospira* biomass from Lake Texcoco to produce dried cakes. Comparable practices have also been reported around Lake Chad in Africa, highlighting the long-standing nutritional and cultural significance of this cyanobacterium [2, 10, 14, 15]. In recent decades, its use in the food industry has expanded considerably, with cultivation being carried out primarily in open raceway ponds, which yield dry powder biomass, and in closed photobioreactors that enable the harvesting of fresh biomass [10, 16–18]. Open systems are economically feasible but face challenges such as temperature fluctuations, contamination, nutrient balance, and production optimization. In contrast, closed systems allow for tight control over variables such as temperature, pH, light intensity, and nutrient concentration, improving yield and biomass composition while minimizing contamination risks [13, 15, 19]. Owing to its simple cultivation and the high nutritional value of the final product, *Limnospira* has become one of the most commercialized “microalgae” worldwide [20].

As a result, *Limnospira* has been incorporated into a wide variety of food products, including energy bars, pasta, bread, cookies, beverages, nutritional supplements, infant formulas, and snacks such as yogurt, cheese, and ice cream [2, 11, 21–24]. The addition of *Limnospira* to these products not only increases their nutritional value but also improves oxidative stability, extends shelf life, and provides a characteristic green color due to the abundance of phycobiliproteins (PBPs), particularly PC [25]. Beyond food applications, *Limnospira* also shows strong potential in the development of functional foods, which are characterized by delivering health-promoting effects that go beyond basic nutrition. Its metabolites have been shown to help reduce blood lipids, combat diabetes and obesity, and

possess antiviral, anti-inflammatory, immunostimulatory, and anticancer properties [2, 7, 8, 21].

Owing to this economic importance, *Limnospira* is one of the most studied cyanobacteria, and its taxonomic classification has experienced multiple revisions over time, persisting into the present. The species within this genus were initially described under *Spirulina* Gomont, such as *S. fusiformis* Voronichin [26] and *S. maxima* Geitler [27], or under *Arthrospira* Gomont, such as *A. platensis* Gomont [28]. In a major taxonomic revision of *Arthrospira*, Komárek and Lund, in 1990 [29], reassigned *S. maxima* and *S. fusiformis* to the planktonic forms of *Arthrospira* (with aerotopes), renaming them *A. maxima* Setchell et Gardner and *A. fusiformis* Komárek et Lund. Later, based on 16S rRNA gene analyses, Nowicka-Krawczyk et al., in 2019 [30], separated these planktonic (with aerotopes) forms of *Arthrospira* into the genus *Limnospira*. More recently, in 2023, Santos et al. [31] revised the taxonomy of *A. platensis*, ultimately transferring this species to *Limnospira* and establishing a reference strain for it.

Taken together, studies on *Limnospira*, its consumption, and incorporation into novel functional food formulations represent promising strategies to address global nutritional demands, promote sustainable diets, and diversify protein sources under climate change and demographic pressures. Moreover, the isolation of new *Limnospira* strains is particularly relevant for sustainable development, especially in developing countries. Accordingly, this study reports a newly isolated *Limnospira* strain from Bolivia and evaluates its nutritional value, highlighting its economic potential as a promising alternative resource.

2. Materials and methods

2.1 Cyanobacteria biomass production and morphological analysis

The strain *Limnospira platensis* LEGE 231238 was isolated in August 2023 from Alalay Lake, Bolivia (17°24'28" S, 66°08'11" W), a hypertrophic brackish water body [32]. Currently, the strain is maintained at the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR LA), Matosinhos, Portugal, in Z8 liquid medium supplemented with 25 g L⁻¹ (Z8₂₅) of Synthetic Sea Salt (Tropic Marine, Berlin, Germany) and 10 µg mL⁻¹ of cyanocobalamin (Kotai, 1972) under the following conditions: 12/12 h (light:dark cycle) with 25 µmol photons m⁻² s⁻¹ and 19°C.

For biomass production, a culture volume of 20 L was prepared using a 1 L inoculum and maintained under continuous aeration for 20 days, with a controlled temperature of 21 ± 2°C, a 16/8 h light/dark photoperiod, and a light intensity of 20 µmol m⁻² s⁻¹ (AsenseTek Lighting Passport, Biosystems, United Kingdom) provided by fluorescent lamps (Arcadia, United Kingdom). Collected biomass was concentrated using a 20 µM nylon mesh (Altmann Analytik, Munich, Germany), and an additional washing step with deionized water was performed to remove salt. The collected biomass was dried in a freeze dryer (Lyoquest-55, Telstar Technologies, S. L., Spain) and stored away from light at - 20°C prior to extract preparation.

For the morphological analysis, LEGE 231238 was observed, microphotographed, and analyzed using the MOTIC Panthera light microscope with a camera and dedicated software (MOTIC Europe, Barcelona, Spain). Thirty cell measurements were performed across various areas of slide preparations.

2.2 DNA extraction, PCR amplification, sequencing, and phylogenetic analysis

Cyanobacterial cells were collected from the culture, and total genomic DNA (gDNA) extraction, PCR amplification, and sequencing were performed following the study by Oliveira et al. [33]. The phylogenomic analyses were conducted according to the study by Hentschke et al. [34], aligning the 16S rRNA gene sequence of LEGE 231238 with *Limnospira* reference strains identified by Santos et al. [31], as well as with other Microcoleaceae.

2.3 Chemical analysis

2.3.1 Extracts preparation

A sequential extraction was carried out from the dry biomass using solvents of different polarities (acetone and water) according to Silva et al. [35] and De Rosa et al. [36], with some modifications. The acetone extract was prepared by mixing 0.5 g of dried biomass with 40 mL of 100% high-performance liquid chromatography (HPLC)-grade acetone. The suspension was sonicated at room temperature in an ultrasonic homogenizer (Sonoplus, probe MS 72, Bandelin, Berlin, Germany) for 5 minutes, with a pulse on for 59 seconds and a pulse off for 3 seconds (amplitude 95%), in order to help break the cell walls and release the cell content. The mixture was centrifuged (Megafuge TM 16 R, Thermo Fisher Scientific, Bremen, Germany) at $5,000 \times g$ at 4°C for 5 minutes, and the extraction process was repeated three times for the same biomass. After completing four extraction sequences, the supernatants were evaporated (BUCHI R-210 Rotary Evaporator, Switzerland). The resulting pellet from the acetone extraction was left to dry overnight in the fume hood. After completely drying, the biomass was extracted with 40 mL of ultrapure water (aqueous extract), following the same procedure as before. The supernatants of the aqueous extract were combined, frozen, and lyophilized (Lyoquest-55, Telstar Technologies, S. L., Spain). The dry extracts (DEs) were kept at -20°C until chemical analysis.

2.3.2 Carotenoid and chlorophyll profiling by HPLC with photodiode array detector

Carotenoid and chlorophyll analyses ($n = 4$) were determined from acetonetic extracts (10 mg mL^{-1}) using a Waters Alliance 2695 HPLC system equipped with a photodiode array detector (PDA, Waters, USA), following a previously described protocol [37] and the methodology described by Oliveira et al. [38], with the following modifications: quantification at 450 nm using external calibration with lutein, zeaxanthin, chlorophyll-*a*, and β -carotene (Extrasynthese, Genay, France). Oxygenated derivatives of β -carotene were expressed as zeaxanthin, β -carotene derivatives as β -carotene, and chlorophyll-*a* derivatives as chlorophyll-*a*. Myxoxanthophyll was identified with an authentic standard solution (DHI, Horsholm, Denmark) and quantified as zeaxanthin. Calibration curves were constructed using four concentrations of standards covering the observed range in samples. Calibration parameters, including r^2 values, limits of detection ($\text{LOD} = 3S_0/b$), and quantification ($\text{LOQ} = 10S_0/b$, where S_0 is the noise standard deviation and b is the calibration slope), are reported in **Table 1**.

Standards	Calibration curve	r^2	LOD ($\mu\text{g mL}^{-1}$) ^a	LOQ ($\mu\text{g mL}^{-1}$) ^b
Lutein	$y = 523141565217x - 1977200435$	0.9930	5.657	17.142
Chlorophyll- <i>a</i>	$y = 52427733333x + 26570$	0.9981	8.909	26.996
Zeaxanthin	$y = 662580827826x + 1302254348$	0.9997	1.253	3.797
β -Carotene	$y = 34727408,0000x - 96390$	0.9992	3.690	11.183

^aLOD: Limit of detection.
^bLOQ: Limit of quantification.

Table 1.
Calibration curves used for pigment quantification.

2.3.3 Spectrophotometric analysis of aqueous extracts

The aqueous extract was dissolved in water to a final concentration of 10 mg mL⁻¹, and PBPs were quantified by measuring absorbance at 562, 615, and 652 nm using a 1cm path length cuvette. This quantification ($n = 4$) was performed and calculated using the following Eqs. 1, 2, and 3 [39]. Similarly, chlorophyll-*a* and total chlorophylls were quantified by measuring the absorbance at 630, 647, 664, and 691 nm (in a quartz cell with 1 cm of optical path), using acetone-water 9:1 to a final concentration of 10 mg mL⁻¹. This quantification ($n = 4$) was performed and calculated using the following Eqs. 4 and 5 [40]. The results were expressed in micrograms of the respective PBP and chlorophyll per milligram of DE.

$$\text{Phycocyanin (PC)} = \frac{A_{615 \text{ nm}} - 0.474 \times A_{652 \text{ nm}}}{5.34} \quad (1)$$

$$\text{Allophycocyanin (APC)} = \frac{A_{652 \text{ nm}} - 0.208 \times A_{615 \text{ nm}}}{5.09} \quad (2)$$

$$\text{Phycocerythrin (PE)} = \frac{A_{562 \text{ nm}} - 2.41 \times \text{PC} - 0.849 \times \text{APC}}{9.62} \quad (3)$$

$$\text{Chlorophyll-}a \text{ (Chl-}a\text{)} = -0.3319 A_{630 \text{ nm}} - 1.7485 A_{647 \text{ nm}} + 11.9442 A_{664 \text{ nm}} - 1.4306 A_{691 \text{ nm}} \quad (4)$$

$$\text{Total chlorophyll (Chl)} = 21.3877 (A_{630 \text{ nm}} + 10.3739 (A_{647 \text{ nm}}) + 5.3805 (A_{664 \text{ nm}}) + 5.5309 (A_{691 \text{ nm}}) \quad (5)$$

2.3.4 Total phenolic content analysis of aqueous extracts

Total phenolic content (TPC) was measured using the Folin–Ciocalteu colorimetric assay, following the method described by Barroso et al. [41], as modified by Morone et al. [42]. Previously, we prepared the gallic acid (GA) solution at 1 mg mL⁻¹ (Sigma, 97.5%–102.5%) as a standard, the Na₂CO₃ (VWR, 99.5%–100.5%) solution at 75 g L⁻¹, and ethanol (for clinical diagnosis, PanReac, AppliChem, ITW Reagents, Barcelona, Spain) at 80%. Calibration curves ($y = 2.7771x - 0.0164$, $r^2 = 0.9998$) were

performed using seven different concentrations of the standard solution. To prepare the blank, in Eppendorf tubes ($n = 4$), we added 25 μL of the standard solution (GA), 25 μL of ethanol (80%), 200 μL of Na_2CO_3 solution, and 500 μL of deionized water. For the Folin reaction, we used Folin–Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) to replace ethanol (80%). For the cyanobacterial strain, the aqueous extract was dissolved in ethanol (80%) to obtain a final concentration of 5 mg mL^{-1} , replacing the standard solution. The incubation and measurement procedures were carried out according to Morone et al. [37]. TPC was expressed as milligram GA equivalents (GAE) per gram of DW. Each assay was performed in four independent replicates ($n = 4$).

2.3.5 Total sugars quantification of aqueous extracts

The determination was performed according to Masuko et al. [43] and DuBois et al. [44], with some modifications. Previously, the phenol solution (20%) (Acros Organic, Geel, Belgium) and glucose monohydrate (1 mg mL^{-1} (Sigma Merck G8270, $\geq 99.5\%$) solution, used as a standard, were prepared. Calibration curves ($y = 1.4616x + 0.0252$, $r^2 = 0.9985$) were generated using six different concentrations of the standard solution. In an Eppendorf tube, we added 100 μL of the standard solution, 500 μL of H_2SO_4 ($\geq 95\%$, Fisher Chemical), and 100 μL of the phenol solution (20%). For the blank, we used ethanol (80%), and for the samples, the aqueous extract was dissolved in ethanol (80%) to obtain a final concentration of 5 mg mL^{-1} , replacing the standard solution. Finally, the microplate was prepared with 200 μL of each Eppendorf tube (blank, standard, sample blank, and samples) and incubated for 30 minutes. Then, the total sugar content was determined by measuring the absorbance at 490 nm with a spectrophotometer (Biotek, Bad Friedrichshall, Germany). The results were expressed in μg glucose equivalents per mg dry extract (DE). The experiment was carried out in quadruplicate ($n = 4$).

2.4 Biomass proximate composition and lipid extraction procedure

Procedures were conducted at Greencolab (<https://www.greencolab.com/>, accessed on 01 April 2025), following the methodology described in Oliveira et al. [38]. The proximate composition of cyanobacterial biomass was determined by quantifying protein, ash, and carbohydrate contents, with carbohydrates estimated by difference from total DW [45]. Lipids were extracted following the Folch method [46, 47], with minor modifications, as described in detail by Oliveira et al. [38]. Lipid content was expressed as % DW using Eq. 6, and results are reported as mean $\pm$ standard deviation ($n = 5$).

$$\text{Lipid content yield} \left(\% \text{ DW} \frac{\text{W}}{\text{W}} \right) = \frac{\text{Weight of the lipid extract (g)}}{\text{Weight of biomass (g)}} \times 100 \quad (6)$$

2.4.1 FA analysis by gas chromatography–mass spectrometry (GC–MS)

FA methyl esters (FAMES) were prepared following the method described in literature [48] and detailed in the study by Oliveira et al. [38]. Results were expressed as mg FA g^{-1} DW, and nutritional quality indexes (AI, TI, and h/H) were calculated according to Eqs. 7–9.

$$AI = \frac{[FA\ 12:0 + (4 \times FA\ 14:0) + FA\ 16:0]}{[\sum MUFA^* + \sum(n-6) + \sum(n-3)]} \quad (7)$$

$$TI = \frac{[FA\ 14:0 + FA\ 16:0 + FA\ 18:0]}{[(0.5 \times FA\ 18:1) + (0.5 \times \sum \text{Other MUFA}) + (0.5 \times \sum(n-6)) + (3 \times \sum(n-3)) + (\frac{\sum(n-3)}{\sum(n-6)})]} \quad (8)$$

$$(h/H) = \frac{[FA\ 18:1\ (n-9) + FA\ 18:2\ (n-6) + FA\ 18:3\ (n-3) + FA\ 20:4\ (n-6) + FA\ 20:5\ (n-3)]}{[FA\ 14:0 + FA\ 16:0]} \quad (9)$$

* Monounsaturated FA

3. Results

3.1 Phylogeny and taxonomy

The ML and BI phylogenetic analyses (see **Figure 1**) placed strain LEGE 231238 within the *Limnospira* clade with strong statistical support (ML = 98, BI = 1). It clustered with the reference strain *L. platensis* CCIBt3254 [31], together with strains *L. platensis* CCIBt3335 and CCIBt3336. The 16S rRNA gene identity between LEGE 231238 and these strains ranged from 99.8% (vs. CCIBt3336) to 100% (vs. CCIBt3254 and CCIBt3335).

Morphologically, strain LEGE 231238 exhibits solitary trichomes with regular coils, not constricted at the cross walls, and lacking a mucilaginous envelope. The spacing between spirals ranges from 30 to 51 μm , while spiral height varies from 25 to 31 μm . Trichome width ranges from 8 to 10 μm , and the cells are shorter than wide, measuring 4–7 μm in length. The apical cells are rounded, often with calyptra. The cell content is blue-green, with numerous aerotopes (see **Figure 2**). The planktonic habit of LEGE 231238, originating from a brackish water body, is consistent with the species' ecology [31].

Based on that, it was evident that the molecular, morphological, and habitat analyses are in agreement and confirm the identification of LEGE 231238 as *Limnospira platensis*.

3.2 Chemical composition analysis

The chemical composition of the biomass, acetonic, and aqueous extract from *L. platensis* LEGE 231238 was characterized. Additionally, data from previous studies with *Spirulina* were included for comparison (see **Tables 2** and **3**). Analysis of the acetonic extracts using HPLC-PDA revealed the presence of several chlorophyll and carotenoid compounds, yielding a good chromatographic resolution, as shown in **Figure 3**. The most abundant pigment was chlorophyll-*a* ($283.53 \pm 11.01\text{ mg g}^{-1}\text{ DE}$) and chlorophyll-*a* derivative ($52.71 \pm 3.95\text{ mg g}^{-1}\text{ DE}$). Among the carotenoids, the most abundant were β -carotene ($45.63 \pm 0.98\text{ mg g}^{-1}\text{ DE}$) and zeaxanthin ($16.56 \pm 1.70\text{ mg g}^{-1}\text{ DE}$). Other carotenoids detected included myxoxanthophyll ($5.43 \pm 0.50\text{ mg g}^{-1}\text{ DE}$), an unidentified xanthophyll ($4.28 \pm 0.24\text{ mg g}^{-1}\text{ DE}$), β -cryptoxanthin ($6.79 \pm 0.60\text{ mg g}^{-1}\text{ DE}$), an unidentified carotenoid ($3.30 \pm 0.01\text{ mg g}^{-1}\text{ DE}$), and a β -carotene isomer ($9.13 \pm 0.80\text{ mg g}^{-1}\text{ DE}$). When considering the total pigment composition, chlorophylls accounted for 78.66% of the total identified pigments, while carotenes represented 16.18%, and xanthophylls 5.11% (see **Figure 4**).

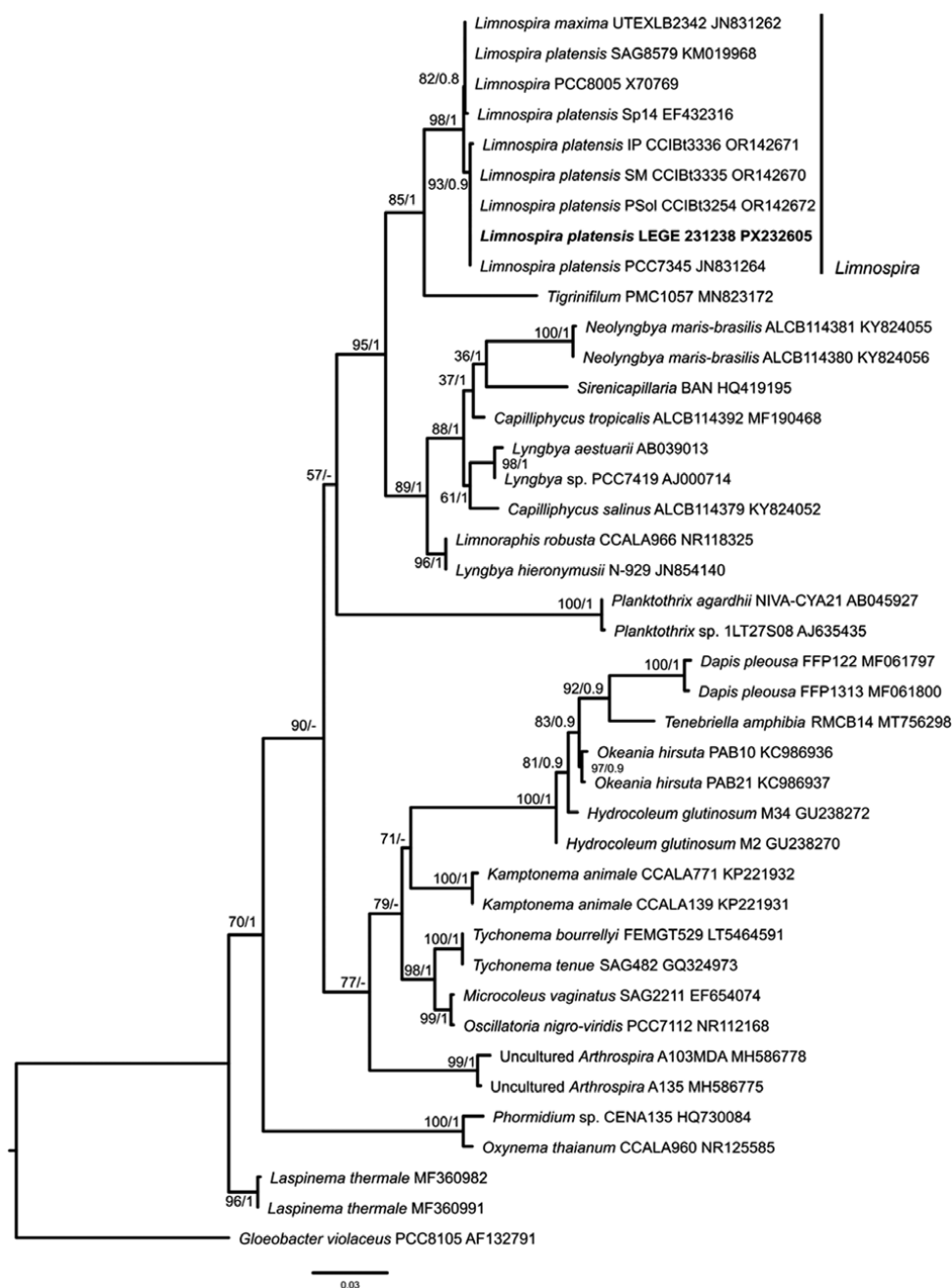


Figure 1.

Phylogenetic tree with 41 sequences, inferred from the 16S rRNA gene using maximum-likelihood analysis. Bootstrap values and posterior probabilities are displayed at the nodes. The strain *L. platensis* LEGE 231238 is in bold.

The aqueous extract was characterized by spectrophotometry for its total phenolic content, total sugars, chlorophylls, and PBPs. The phenolic content was $15.64 \pm 1.76 \mu\text{g}$ of GAE mg^{-1} of DE, and the total sugar content was determined to be

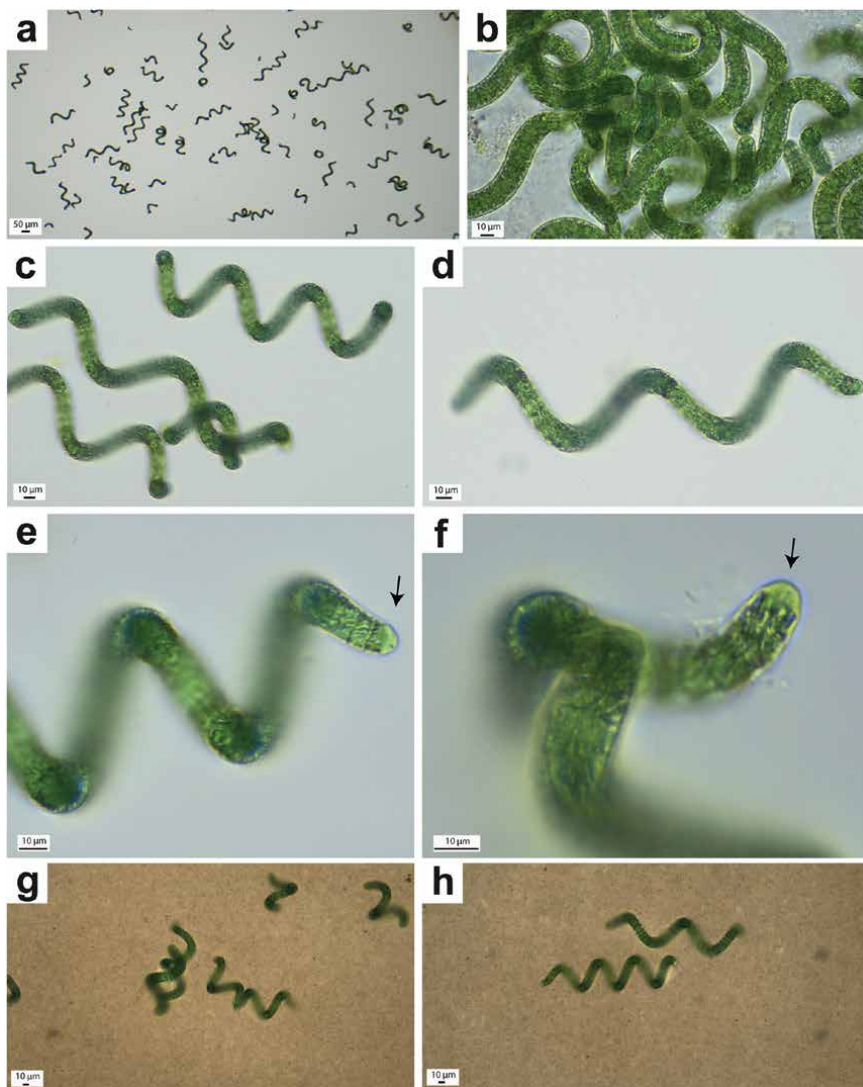


Figure 2. Microphotographs of *L. platensis* LEGE 231238. (a) General overview of trichome arrangement; (b) general overview showing trichomes with aerotopes; (c, d) details of trichome coiling; (e, f) trichomes with calyptra, pointed out by the black arrows and aerotopes; (g, h) Slides prepared with China ink, showing the lack of mucilaginous envelopes.

$7.19 \pm 0.75 \mu\text{g}$ of glucose equivalents mg^{-1} of DE (based on the calibration curve obtained in the assay). Regarding the chlorophyll-*a* and total chlorophylls content, the values were 5.84 ± 1.87 (3.4%) and 10.02 ± 2.06 (5.8%) $\mu\text{g mg}^{-1}$ of DE, respectively. The PBPs profile revealed concentrations of $84.88 \pm 8.29 \mu\text{g mg}^{-1}$ of DE for PC (48.8%), $53.11 \pm 6.21 \mu\text{g mg}^{-1}$ of DE for APC (30.5%), and $20.25 \pm 3.12 \mu\text{g mg}^{-1}$ of DE for PE (11.6%).

The proximate composition of cyanobacterial biomass revealed a high protein content, with $45.40 \pm 0.54\%$ of DW. Carbohydrates were the second most abundant component, with $27.84 \pm 0.25\%$ of DW, followed by ash content with $19.90 \pm 0.32\%$

	<i>L. platensis</i> LEGE 231238	<i>Spirulina</i> sp. [2]	<i>S. platensis</i> [9]	<i>A. platensis</i> [49]	<i>Spirulina</i> sp. [50]	<i>S. platensis</i> [48]	<i>Spirulina</i> [51]
Total protein	45.4	57.5	46–63	23.8–62.9	48.2	67.8	62.8
Total lipids	6.9	7.7	4–9	1.5–8.1	5.3	1.6	6.9
Carbohydrates	27.8	23.9	8–14	3.6–15.8	14.2	7.1	–
Ash	19.9	6.2	–	8.2–18.11	10.1–14.6	11.7	7.5

The values are expressed in percentage (%).

Table 2.
Macronutrient profile of different Spirulina strains.

	<i>L. platensis</i> LEGE 231238	<i>Spirulina</i> sp. [47]	<i>S. platensis</i> [52]	<i>A. platensis</i> [49]
FA 14:0	1.23	0.1	0.28	0.42–3.65
FA 16:0	39.05	38.6	42.85	43.60–71.15
FA 16:1	1.45	–	–	–
FA 16:1 <i>n</i> –7	6.82	5.1	3.16	4.36–7.60
FA 18:0	0.96	5.4	1.00	0.69–3.33
FA 18:1 <i>n</i> –9	3.10	2.6	3.53	4.02–5.99
FA 18:1	1.16	–	–	–
FA 18:2 <i>cis</i> (<i>n</i> –6)	14.92	21.4	14.43	7.86–20.15
FA 18:3 <i>n</i> –6	31.32	23.3	22.45	2.73–4.50
AI	0.75	0.7	0.89	–
TI	1.32	1.6	0.28	–
PUFA	46.23	44.8	40.6	10.59–24.52
SFA	41.24	44.2	50.1	62.94–80.31
MUFA	12.53	10.9	8.5	9.11–12.58
<i>n</i> –3 PUFA	–	–	2.9	0.01–0.20
<i>n</i> –6 PUFA	46.23	44.6	37.7	10.59–24.48
h/H	0.45	0.6	1.0	–

Values are expressed in relative abundance (%) and represent the mean of five analytical samples.

Table 3.
*Fatty acid profile identified in the total lipid extract of the cyanobacteria strain *L. platensis* LEGE 231238.*

of DW and lipid content with $6.86 \pm 0.18\%$ of DW. Elemental CHN analysis showed that carbon (C) represented $38.83 \pm 0.28\%$ of DW, hydrogen (H) $6.47 \pm 0.08\%$ of DW, and nitrogen (N) $7.26 \pm 0.09\%$ of DW, and the C:N ratio was $5.54 \pm 0.17\%$ of DW (see **Figure 4**). These values are in agreement with data from previous studies on *Spirulina* strains, as shown in **Table 2**.

The FA composition of the lipid extract was determined to assess its lipid profile and potential nutritional and biotechnological applications. This analysis allowed the identification of nine FAs across *L. platensis* LEGE 231238 (see **Figure 5**) and revealed a predominance of saturated FA (SFA) with $41.24 \pm 0.72\%$ and

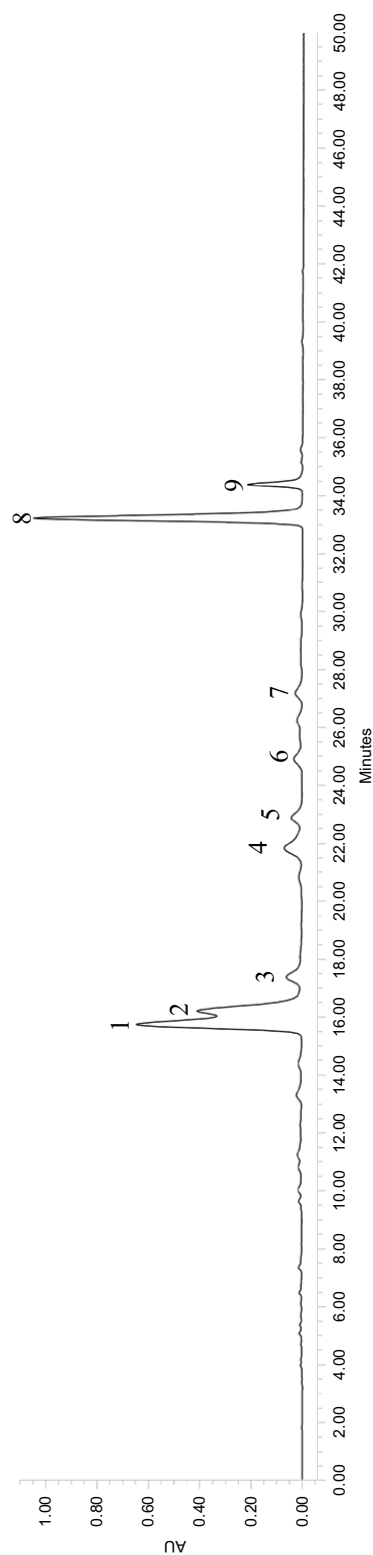


Figure 3. Carotenoid and chlorophyll profiles of the acetonic extract of *L. platensis* LEGE 231238 obtained by high-performance liquid chromatography with photo-diode array detection (HPLC-PDA), recorded at 450 nm. Chlorophyll-a (1), zeaxanthin (2), chlorophyll-a derivative (3), myxoxanthophyll (4), unidentified xanthophyll (5), β -cryptoxanthin (6), unidentified carotenoid (7), β -carotene (8), and β -carotene isomer (9).

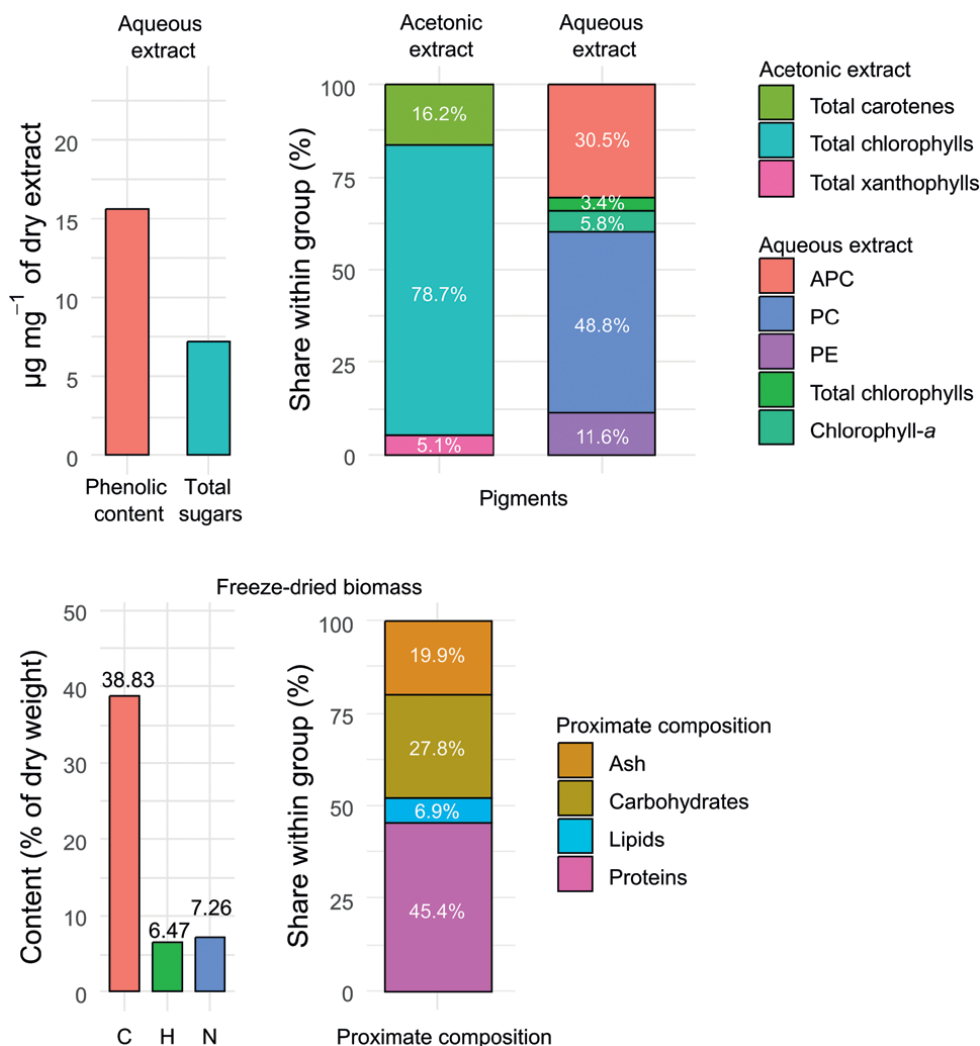


Figure 4.

Graphical representation of the composition and relative distribution of the analyzed groups. Bar charts on the left show absolute abundance, while stacked bar charts on the right display the percentage contribution of each group. Colors represent different categories.

polyunsaturated FA (PUFA) with $46.23 \pm 0.60\%$. The most abundant FA was palmitic acid (FA 16:0), representing $39.05 \pm 0.66\%$, followed by γ -linolenic acid (FA 18:3 $n-6$) with $31.32 \pm 0.30\%$ and linoleic acid (FA 18:2 $cis\ n-6$) with $14.92 \pm 0.73\%$. Among the MUFAs ($12.53 \pm 0.50\%$), palmitoleic acid (FA 16:1 $n-7$) was the most abundant ($6.82 \pm 0.12\%$), followed by FA 16:1 and 18:1 ($1.45 \pm 0.12\%$ and $1.16 \pm 0.13\%$, respectively). Minor constituents were also identified, such as oleic acid (FA 18:1 $n-9$) with $3.10 \pm 0.27\%$, myristic acid (FA 14:0) with $1.23 \pm 0.08\%$, and stearic acid (FA 18:0) with $0.96 \pm 0.08\%$. The potential health benefits of the cyanobacterial strain were also determined by AI ($0.75 \pm 0.03\%$), TI ($1.32 \pm 0.04\%$), and h/H index ($0.45 \pm 0.03\%$).

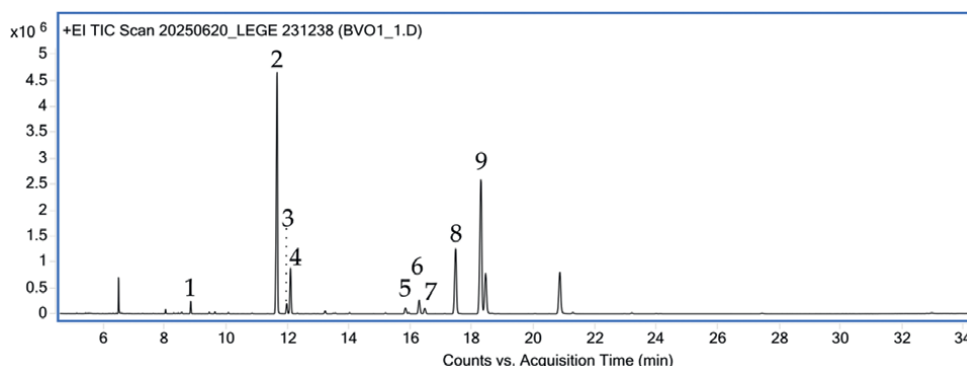


Figure 5.
 FAs profile of the lipid extract of *L. Platensis* LEGE 231238 obtained by gas chromatography–mass spectrometry (GC–MS). FA 14:0 (1), FA 16:0 (2), FA 16:1 (3), FA 16:1 n–7 (4), FA 18:0 (5), FA 18:1 n–9 (6), FA 18:1 (7), FA 18:2 *cis* n–6 (8), and FA 18:3 n–6 (9).

4. Discussion

The growing market interest in sustainable and alternative food sources, nutritional supplements, and bioactive compounds has positioned cyanobacteria at the forefront of research. Spirulina is renowned for its rich and diverse biochemical composition, particularly protein, carbohydrates, pigments, and essential lipids, such as *n*–6 FA, which underscore its importance as a nutritious and sustainable dietary supplement [53].

To understand the nutritional potential of *L. platensis* LEGE 231238, it is essential to evaluate the biomass composition, thereby providing an overview of its biochemical profile. Carbon and nitrogen metabolism are essential for biological systems, as they are involved in the synthesis of energy carriers, proteins, and pigments, and are tightly interconnected in various organisms. In plants and prokaryotes, this coupling is primarily driven by two main factors [54–57]. First, carbon (C) and nitrogen (N) are among the most abundant elements in cells, requiring coordination to avoid metabolic inefficiencies. Secondly, nitrogen assimilation depends on the availability of C skeletons for biosynthesis; therefore, a deficiency or excess of one element significantly influences the metabolism of the other [57]. Such regulation can occur at multiple levels, from allosteric control of nutrient assimilation proteins to mechanisms regulating the expression of their genes [58–60]. Hydrogen content (H) is typically used to determine the calorific value, which reflects its energy potential [61].

These CHN values observed in *L. platensis* LEGE 231238 are consistent with the typical ranges reported for cyanobacterial biomass and reflect a high nutritional value [62]. The elevated N content supports the high protein level observed and reinforces its potential as a sustainable source of bioactive and nutritional compounds. Proteins are the primary nitrogen-containing biomolecules in microalgae and cyanobacteria. Furthermore, the C:N ratio was approximately $5.35 \pm 0.2\%$, which is indicative of protein-rich biomass [63]. A low C:N ratio is generally characteristic of nitrogen-rich cells and is considered desirable for nutritional and biotechnological applications, particularly in the food and feed industries [63].

Regarding that, *Spirulina* is globally recognized for its high protein content and nutritional value. When comparing the protein content obtained herein ($45.40 \pm 0.54\%$) to other cyanobacterial genera, such as *Spirulina* [2], *Anabaena*, and *Aphanizomenon* [9], with values of 57.5%, 43%–56%, and 62%, respectively, we notice that, even being in a similar range, the protein content of *L. platensis* LEGE 231238 was slightly lower. However, the protein content of this strain is within the *Spirulina* range. Aouir et al. [49] demonstrated that *L. platensis* (identified as *Arthrospira platensis*; in powder and lyophilized form) from different countries and cultivated in different media has a protein content ranging from 23.78% to 62.86%. Another study conducted by Rodriguez de Marco et al. [48] identified the protein from *L. platensis* (as *S. platensis*) from Cuba with $67.82 \pm 1.71\%$. In general, it seems that the protein content is correlated to cultivation conditions, as well as different culture media [49].

Another important parameter is the lipid content, as it is key to evaluating the nutritional potential of cyanobacterial biomass. *Spirulina* has been widely recognized for its potential health benefits, particularly in the context of obesity and metabolic disorders [64]. Preclinical and clinical studies have demonstrated its efficacy in improving various health parameters, such as reducing body weight, blood pressure, and plasma triglycerides, while also exhibiting antioxidant and cardiomyocyte-protective activity [4, 65, 66]. From a nutritional perspective, *Spirulina* is particularly valued for its FA profile, especially as a rich source of *n*-6 PUFAs. These FAs cannot be synthesized by the human body and must be obtained through the diet [67]. While most modern diets are deficient in *n*-6 PUFAs, the inclusion of supplements like *Spirulina* can help rebalance lipid intake [68]. *Spirulina* has been identified as one of the best whole food sources of *n*-6, offering an accessible option for dietary supplementation [69, 70]. Given the alarming global rise in obesity-related diseases, with millions of deaths and significant burdens on public health systems, the incorporation of *Spirulina* into daily nutrition may offer a promising and natural strategy to support weight management and improve metabolic health [4, 71, 72].

In our study, *L. platensis* LEGE 231238 showed similar lipid content (6.86%) to the commercial *Spirulina*, as described in **Table 2** (1.5%–8.1%). Differences in lipid content among the cyanobacteria are influenced by diverse factors, as reported in the study with *Synechocystis* sp. PCC 6803 [73], also including nutrient availability, light intensity, and oxidative stress, among others. It is important to note that total lipid levels can vary between species [9] and are also affected by the extraction method applied [74]. Conde et al. [47] identified in *Limnospira* sp. (as *Spirulina* sp.) a higher lipid content (10.7% of biomass) when compared to our cyanobacterial strain. The total PUFA content in our strain (46.23%) also agrees with the typical ranges reported for *Limnospira* (as *Spirulina*), in a study conducted by Conde et al. and Selina et al. [47, 52]. Another study by Aouir et al. [49] identified lower PUFA content (10.59%–24.52%) in *L. platensis* (as *A. platensis*). Notably, in some studies, the PUFA profile was entirely dominated by *n*-6 FA, with no detectable *n*-3 PUFAs [47, 49].

Nutritional indices such as the AI and TI are valuable parameters for assessing the potential health effects of dietary lipids [75]. In our study, the values of AI (0.75%) and TI (1.32%) are similar (see **Table 3**) to those reported in commercial *Spirulina* [47, 52]. Although the TI was moderately high, this is expected due to the elevated SFA content, particularly palmitic acid, which is the most abundant in our cyanobacterial strain. The *n*-6/*n*-3 PUFA ratio was not possible to calculate, given

the absence of detectable $n-3$ PUFAs. The PUFA/SFA index is used to evaluate the nutritional value of foods and the impact of diet on cardiovascular disease, with PUFAs having a beneficial effect on health, whereas SFAs have a positive impact on high cholesterol levels [76, 77]. *L. platensis* LEGE 231238 demonstrated a similar PUFA/SFA index value (1.12%) to those reported for *Sargassum tenerrimum* (1.18%) [78], *Concholepas concholepas* (1.16%) [79], *Scomberomorus cavalla* (1.18%) [80], and chicken (0.308–2.042%) [81]. The PUFA/SFA index of *L. platensis* LEGE 231238 indicates a favorable FA profile, since high PUFA/SFA ratios are generally associated with improved nutritional quality and potential health benefits, particularly in reducing the risk of cardiovascular diseases [82, 83].

In another biotechnological context, *Spirulina* is renowned for producing a broad spectrum of bioactive compounds, such as chlorophylls, carotenoids, PBPs, phenolic compounds, and carbohydrates, each with valuable nutritional and biotechnological applications. Actually, there is a high demand for antioxidant compounds as food additives to help protect against oxidative stress and reduce the risk of developing non-communicable diseases (NCDs) [84].

L. platensis LEGE 231238 exhibited a remarkable pigment profile, with high levels of chlorophyll-*a* ($283.53 \pm 11.01 \text{ mg g}^{-1} \text{ DE}$) and β -carotene, surpassing values previously reported for cyanobacteria [85]. Chlorophyll-*a*, the dominant photosynthetic pigment, plays a pivotal role not only in photosynthesis but also in human health, demonstrating antioxidant, anti-inflammatory, lipid-reducing, and even potential anti-obesogenic and antimutagenic activities [86, 87]. Carotenoid dietary intake and their accumulation in tissues have been positively correlated with a lower risk of chronic diseases, for which the recognized antioxidant activity of these compounds strongly contributes. β -carotene is the predominant carotenoid in the human diet, serum, and human body tissues, serving as the main source of vitamin A for humans. This carotene has a disease-protective role, being inversely correlated with the risk of ophthalmic, metabolic, and cardiovascular diseases and certain cancers [88–90]. On the other hand, zeaxanthin, together with *meso*-zeaxanthin and lutein, is a macular carotenoid, recognized for its importance in the protection of the retina, possibly preventing and/or slowing the progression of blindness [91].

Nevertheless, it is not always possible for the general population, especially the elderly, to consume adequate amounts of these pigments daily through fruits and vegetables. For this reason, the inclusion of superfoods like *Spirulina* in the human diet can be advantageous in terms of the stability and bioavailability of bioactive compounds of interest for human health.

PBPs are water-soluble pigment proteins that play a crucial role in harvesting light for photosynthesis in cyanobacteria, and the major types found in *Spirulina* include PC, APC, and PE [92, 93]. PC is the most abundant and extensively studied due to its distinctive blue color and growing interest in the food, cosmetic, pharmaceutical, and diagnostic industries [49, 94, 95]. C-PC, a PBP derived from *Aphanizomenon flos-aquae*, has been utilized in the formulation of NaturCyanin Bioactive® (CAS No. 11016–15-2) as a natural blue colorant alternative to synthetic dyes. PC has also demonstrated significant antioxidant properties and protective effects against oxidative stress-induced cellular damage [96]. APC is commonly used as a fluorescent marker due to its strong far-red emission and quantum efficiency [97]. PE, similarly to PC, is used as a pink colorant and has applications in fluorescence microscopy and imaging [98]. Our findings show that

the values of PC, PE, and APC (84.88%, 20.25%, and 6.21%, respectively) are higher than those reported by [49], where *A. platensis* was identified with PC (1.80%–8.70%), APC (1.10%–7.20%), and PE (1.30%–3.3%). Kumar (2011) [78] also identified PC (5.38%–7.73%), APC (2.58%–3.46%), and PE (0.63%–1.80%). When *L. platensis* LEGE 231238 is compared with another cyanobacterial genus, such as *Pseudanabaena tenuis*, the values are different for PC (2%), APC (9%), and PE (89%) [96]. Thus, *L. platensis* LEGE 231238 is worth further study regarding its biological activities and safety profile, highlighting its application in the food, feed, and pharmaceutical industries for the sustainable production of bioactive compounds.

In addition to the antioxidant and protective effects of PBPs, *Spirulina* also contains other classes of bioactive compounds that contribute to its functional potential. Among them, phenolic compounds have garnered increasing attention due to their potent antioxidant activity [42, 99]. These metabolites act by scavenging reactive oxygen species and preventing oxidative damage to biomolecules, thereby supporting cellular defense systems [100]. *L. platensis* LEGE 231238 showed a phenolic content of $15.64 \pm 1.76 \mu\text{g}$ of GAE mg^{-1} DE. These values are lower than those reported by Aouir et al. [49] for *A. platensis*, which ranged from 19.61 to 67.52 mg GAE g^{-1} of DW. This difference may be attributed to variations in the methodologies used for the extraction of phenolic compounds. However, when the same extraction and quantification methodology was applied to different genera of cyanobacteria (1.07 to 2.45 μg of GAE mg^{-1} of DE) [42], the phenolic content observed in *L. platensis* LEGE 231238 was higher. El-Baky [101] demonstrated that the phenolic content in *S. maxima* ($12.94 \pm 0.93 \text{ mg GAE g}^{-1}$) significantly correlates with its radical scavenging capacity, reinforcing the importance of these compounds in the overall bioactivity of cyanobacterial products.

Other water-soluble metabolites are present in the aqueous extract, such as total sugars, which also contribute to the nutritional and functional value of *L. platensis* LEGE 231238. Carbohydrates play a vital role in human physiology, serving as a primary energy source, regulating blood glucose and insulin metabolism, and contributing to cholesterol and triglyceride metabolism [102]. The determination of total sugar content provides a comprehensive assessment of the glycidic profile of the aqueous extract, which is crucial for the development of dietary supplements and functional products derived from cyanobacteria. The total sugar content observed in *L. platensis* LEGE 231238 was $7.19 \pm 0.75 \mu\text{g}$ of glucose equivalents per mg of DE.

In conclusion, this study confirmed the classification of strain LEGE 231238 as *L. platensis* based on both molecular and morphological analyses. The nutritional profile of this strain is comparable to that of other *Spirulina* strains currently available on the market, supporting its suitability as a food supplement. Notably, this work represents the first published report of an *L. platensis* isolate from Bolivia and underscores its strong potential for commercial exploitation. Future studies on this strain should focus on optimizing cultivation conditions and enhancing the production of its bioactive compounds.

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Conflict of Interest

The authors declare that there are no financial or non-financial competing interests that could be perceived as influencing the work reported in this chapter.

Consent to publication

All authors have explicitly agreed to the publication of this chapter.

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Limno-Ecological Changes That Influenced Peridinium Decline and Cyanobacteria Enhancement in Lake Kinneret, Israel

Moshe Gophen

Abstract

Lake Kinneret is the only natural freshwater lake in Israel. The lake and its headwaters are utilized for approximately 26% of the national water budget, including supply, commercial fishery, recreation, and tourism. The water quality of Lake Kinneret is, therefore, a national concern. The area of the Kinneret drainage basin is 2,730 km² of which the entire Hula Valley is about 200 km². Three major headwater rivers flow southward and jointly create the Jordan River, which then flows into Lake Kinneret. The Jordan River contributes approximately 63% of the Kinneret water budget and more than 50% of the total external nutrient inputs, which are critical for algal growth. In the 1950s, the wetland and old Lake Hula were drained, and the land was converted from a natural ecosystem habitat to agricultural development. Before drainage, the Hula wetlands area was densely covered by submerged and emergent aquatic vegetation, and its water cover was mostly anoxic, containing ammonium, with no nitrates detected. Until the late 1990s, the Kinneret phytoplankton communities were predominantly dominated by Pyrrophyta (*Peridinium* sp.), but later, cyanobacteria took over, leading to deteriorating water quality. The historical record of the N₂-fixing toxic cyanobacterium, *Aphanizomenon ovalisporum*, blooming signaled a warning of water quality deterioration. Evaluation of long-term data records of lake and watershed nutrient dynamics investigates the upheaval of phytoplankton assemblage composition change in the lake.

Keywords: Kinneret, Hula, N-insufficiency, P-sufficiency, peridinium, cyanobacteria

1. Introduction

A 10-year (1969–1979) non-modulated dynamical comprehensive structure of the Kinneret ecosystem was documented, entitled “Model A” [1]. Twenty-five years later, a modulated evaluation of a 32-year (1969–2001) dynamical comprehensive structure Lake Kinneret database, with a scoped focus on the role of zooplankton, was documented, entitled “Model B” [2]. Although there is a partial dissimilarity between the two documents, there is a conceptual overlap between them. The usage

of metabolic and geochemical ecological coefficients measures interaction rates between ecosystem compartments. Model B includes temperature distribution partitioning, and Model A emphasizes the role of fish [3–6]. Model B illustrates the potentially useful information to complement field data collection with respect to the role of zooplankton in nutrient cycles. Model B is coupled to physical modulation, whilst Model A relies solely on food web dynamics as thermally affected. Model B quantifies the role of zooplankton in the distribution and flux of Carbon, whilst Model A illustrates the carbon flow pattern throughout all ecosystem compartments. The two models do not illustrate predictive instructions for management design aimed at preventing deterioration or improving water quality in Lake Kinneret, although fishery management through the removal of unwanted zooplankton consumer fishes is implied in Model A [1, 3, 4, 7, 8]. The impact of nutrient dynamics and composition on water quality is not concluded in either model. The objective of this paper is to evaluate the temporal dynamics of internal and external nutrient input with respect to climate conditions. The consequences on the structure of phytoplankton communities and the process of Pyrrhophyte decline and enhancement of cyanobacteria are analyzed.

During the summer of 1994, a bloom of the harmful cyanobacterium (HFCB), the predictable *Aphanizomenon ovalisporum* (AO), was recorded in Lake Kinneret for the first time since the early 1960s [3, 9–12]. Therefore, the Lake Kinneret ecosystem could be classified, like many other lakes, as a potential HFCB ecosystem, where insufficient nitrogen is accompanied by phosphorus sufficiency, causing a decline in the N/P mass ratio [9–16]. The documented ecological parameters that enabled the first historical outbreak of the N₂-fixing cyanobacterium, AO, were widely studied [10–13, 17–18]. Despite several studies relevant to eco-limnological changes in the Lake Kinneret ecosystem [3–6, 8, 9, 12, 20, 21], a comprehensive ecological retrospective and future prediction of the entire ecosystem, including the watershed and the lake as an integrated system, along with consequent management design, is missing. The objective of this paper is to fill this knowledge gap from a management perspective.

Lake Kinneret and its watershed, including the Hula Valley, are located in northern Israel's Syrian–African Great Rift Valley. Lake Kinneret is the only natural freshwater lake in Israel. Between 1970 and 2010, an annual average of 336 mcm (10⁶ m³) of Kinneret water was withdrawn from the lake and supplied to the south for domestic and agricultural irrigation usage. The water quality of Lake Kinneret is, therefore, a national concern. The total national water supply is 2.11 bcm (10⁹ m³), of which 0.55 bcm comes from the Kinneret–Jordan hydrological system. The area of the Kinneret drainage basin is 2,730 km² and is located mostly to the north of the lake, with the total Hula Valley covering about 200 km². Three major headwater rivers (Hatzbani, Banyas, and Dan) flow from the Hermon Mountain region in the north, join together, and form the Jordan River, which crosses the Hula Valley and flows into Lake Kinneret. Until 1957, the Jordan crossed the Hula Valley through three branches (tributaries) flowing into the old Lake Hula. From Lake Hula, at an altitude of 61–68 masl (meters above mean sea level), the Jordan River flows downstream into Lake Kinneret (mean water level 211 mbsl) for a distance of approximately 15 km. The altitude difference between the top of Mount Hermon (2,814 masl) and Lake Kinneret's water level (mean 211 mbsl) along a spatial distance of 70 km results in a mean slope of 4.3%, creating a rather strong erosive force. The Jordan River contributes approximately 63% of the Kinneret water budget and more than 50% of the total external nutrient inputs. Until

1957, the Hula Valley land was covered by old Lake Hula (1.5 m mean depth; 13 km² water surface) and 3,500 ha of swampy wetlands. The wetland area was covered by water in the winter and partly covered in the summer. To the north of the swamps was an area (3,200 ha) where water table levels were high in winter, thus making agricultural cultivation impossible. During the summer, when underground water levels declined, this 3,200-ha land surface was efficiently cultivated. The wetland area was densely covered by submerged and emergent aquatic vegetation, and the water cover was mostly anoxic, containing ammonium, with no nitrate detected [22].

The objective of this study is to reveal the ecological background that created the current development. The unique bloom outbreak of AO in the summer of 1994, the depleted biomass density of the routinely formed *Peridinium* bloom, and the enhancement of cyanobacteria instead were widely investigated and documented. The impact of external and internal nutrient inputs that induced those changes is the focused narrative of the present study. The skeletal concept of this paper is not the highly documented information about the resulting conditions, but the background induction of it. Lake conditions maintained during the occurrence of the AO outbreak were intensively investigated. On the other hand, the tentative factors of the environmental background that induced the enhancement of cyanobacteria were not thoroughly studied and are the focus of this paper.

2. Materials and methods

2.1 Data sources

The data about nutrient concentrations and phytoplankton density were taken from the Kinneret Limnological Laboratory Data Base and Annual (1969–2001) reports [23] (M. Schlichter). Annual (1969–2001) and monthly data of rainfall capacities measured at the Dafna (northern part of the Hula Valley) meteorological station were obtained from the Israeli Meteorological Service (courtesy of M. Peres). Nitrogen and phosphorus concentrations in River Jordan (Huri Station) were taken from Annual (1967–2001) reports.

2.2 Kjeldahl (organic nitrogen plus ammonia)

Total-unfiltered sample; dissolved-filtered sample; digestion – H₂SO₄; distillation – NaOH; titration – ammonia.

Total nitrogen (TN), nitrate (NO₃): Diphenylamine sulfuric acid chromogen – NAS method.

Ammonia (NH₄): Phenol-hypochlorite method.

Total phosphorus (TP) and total dissolved phosphorus (TDP): Persulfate oxidation/ascorbic acid method.

2.3 Statistical evaluation

Statistical evaluation was conducted using the software STATA 17.0 – Standard Edition, Statistics and Data Science, Copyright 1985–2021 StataCorp LLC, StataCorp, 4905 Lakeway Drive, 800-STATA-PC. The Stata license is a single-user perpetual license, serial number: 401706315938, licensed to Moshe Gophen, Migal. Three

statistical methods were utilized: Linear and fractional polynomial (with 95% confidence limits), regression (prediction), and biplot PCA multivariate analysis.

3. Results

3.1 Monthly distribution of nutrient concentrations in Jordan water

The monthly and temporal distribution of nitrogen, phosphorus, and the TN/TP ratio in the Jordan water (Huri Station) are shown in the following figures and tables.

Results given in **Figures 1–3** confirm the *Peridinium* response to TN enrichment by division rate and biomass density enhancement during winter, spring, and early summer, and biomass decline during mid-summer and fall in response to TN diminishment. The TN periodical increase starts earlier than the TP elevation. Previous studies [1, 13, 17, 21, 24–26] confirmed *Peridinium* TP-mediated biomass transportation factor from bottom sediments into the epilimnion, whereas TN epilimnetic sources are mostly external and partly derived from ammonium oxidation. Consequently, the fall-winter increase of the TN load in the epilimnion initiates earlier than that of TP when the destratification process progresses. The *Peridinium* bloom intensity is dependent on the dynamics (rate and range) of nitrogen import from the catchment through headwater flows. The higher the nitrogen, the denser the *Peridinium* biomass. However, despite the pattern similarity of nitrogen and phosphorus external import, the *Peridinium* bloom biomass density does not depend on epilimnetic phosphorus availability. Moreover, the *Peridinium* bloom is initiated by the excystation process of cysts within bottom sediments, transforming into a movable form equipped with a flagellum. These vegetative mobile cells are phosphorus-rich, having incorporated phosphorus from the sediment substrate. The bloom development is promoted by an intensive division

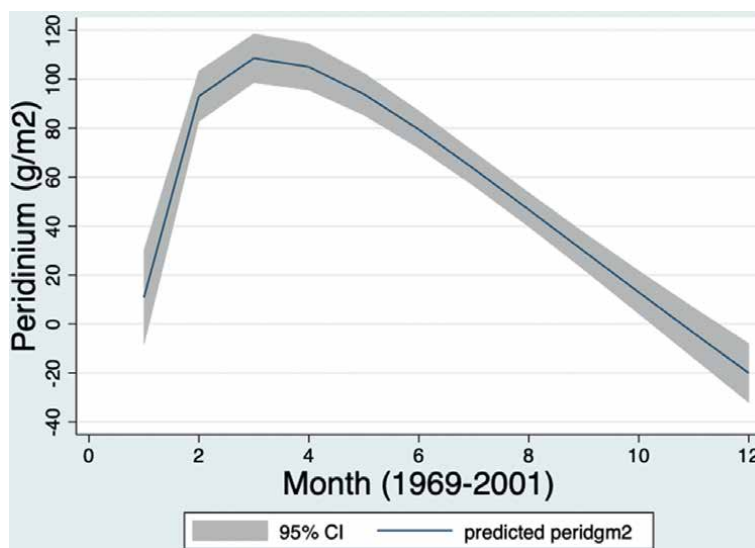


Figure 1. Fractional polynomial (with 95% confidence limit) correlative regression plot between monthly means of Pyrrhophyta (*Peridinium* spp.) biomass ($\text{g(w)}/\text{m}^2$) and month in Lake Kinneret during 1969–2001.

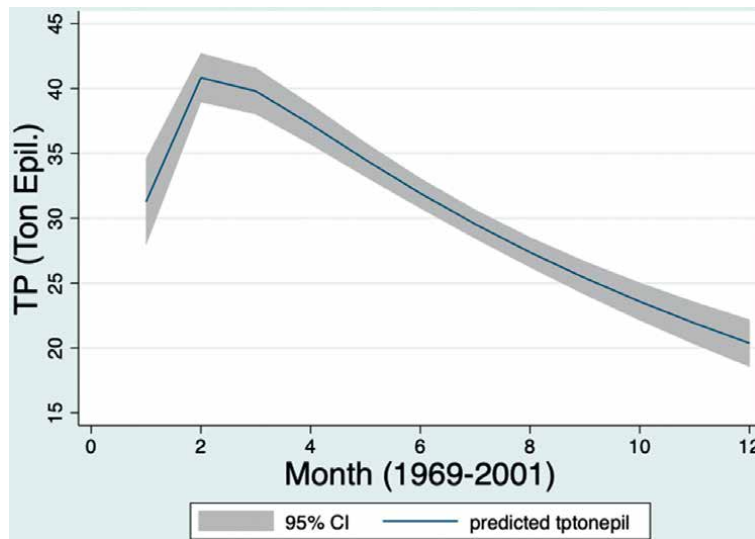


Figure 2. Fractional polynomial (with 95% confidence limit) correlative regression plot between monthly means of total phosphorus (TP) epilimnetic load (tons) and month in Lake Kinneret during 1969–2001.

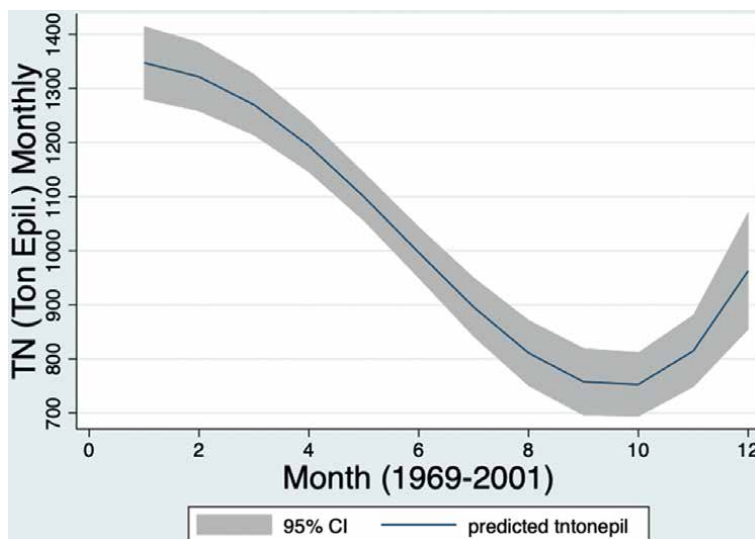


Figure 3. Fractional polynomial (with 95% confidence limit) correlative regression plot between monthly means of total nitrogen (TN) epilimnetic load (tons) and month in Lake Kinneret during 1969–2001.

rate, creating a factor of epilimnetic phosphorus enhancement. *Peridinium* cells' natural mortality, degradation, and decomposition induce the release of dissolved organic phosphorus (DIN) to the epilimnion. The bloom onset starts in late winter and offsets occur during early summer (June) [1, 27]. This phenomenon sequence of a winter-heavy *Peridinium* onset bloom, terminated by early summer with intensive

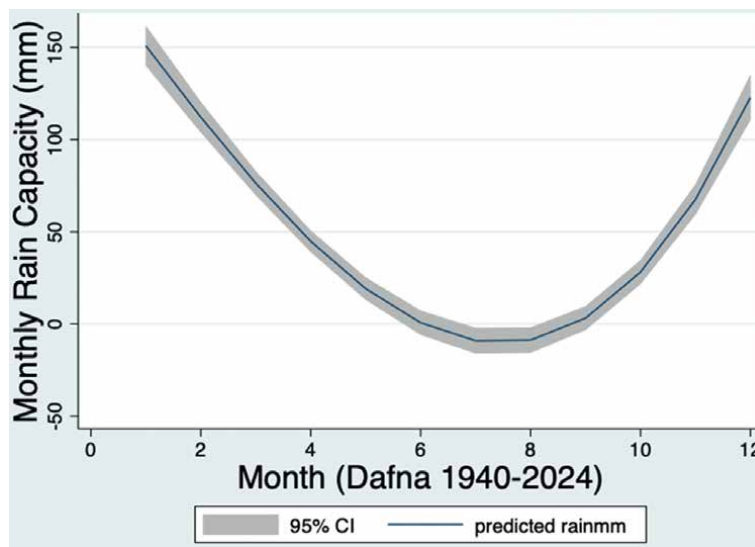


Figure 4. Fractional polynomial (with 95% confidence limit) correlative regression plot between monthly means (1940–2024) of rainfall capacity (Dafna) and month.

cyst formation (cystation), followed next year by epilimnetic high densities of vegetative bloom inset, and consequently bloom offset collapse and high DIN concentration in early summer, enabled the outbreak of AO. Moreover, these complex interactions between climate conditions, nutrient dynamics, and cyanobacteria in Lake Kinneret are a repeatable situation. These conditions are suitable for N_2 -fixer cyanobacterium blooming: Low nitrogen, sufficient phosphorus, and diminished competitor *Peridinium* biomass. What else is conceivable for the HFCB outbreak? A reasonable inoculum.

Results shown in **Figures 3 and 4** indicate the correlation between rainfall, river discharges, and the epilimnetic nitrogen load, highlighting the impact of externally sourced nitrogen input. River discharge plays a role as a transporter in the Kinneret ecosystem, with the transport load range being significantly influenced by rainfall capacity and flow rate. Typically, high loads occur in winter and low loads in summer. However, the transport range depends on the availability of nutrient sources. Erosion caused by water flow friction on the landscape's rocky infrastructure is greatly affected by the natural discharge range. Nonetheless, the contribution of anthropogenic resources, such as human and domestic dairy sewage (raw and treated) or fishpond effluents, is independent of the natural rainfall-discharge regime. Before the Hula drainage, nitrogen input loads into Lake Kinneret, mainly as ammonium, were significantly increased by *Peridinium*. After the Hula drainage, ammonium influx from the wetlands was replaced by peat soil components and nitrate, which has a lower affinity for *Peridinium*. However, this shift is complicated by sewage and fishpond effluents. Since the late 1980s, following the removal of domestic and dairy sewage and the conversion of fishponds to field crops, organic nitrogen input was reduced [28], leading to a period of N-limitation and a decline in the N/P ratio. These conditions favored cyanobacteria over *Peridinium*, creating a less suitable environment for their growth.

3.2 Temporal (1970–2001) distribution of nutrient concentrations in Jordan water

Results shown in **Figure 5** represent the correlation between TN and TP epilimnetic load sizes ordered from small to large. **Figure 5** emphasizes the impact of external and internal, different or similar properties, on TN and TP. Nevertheless,

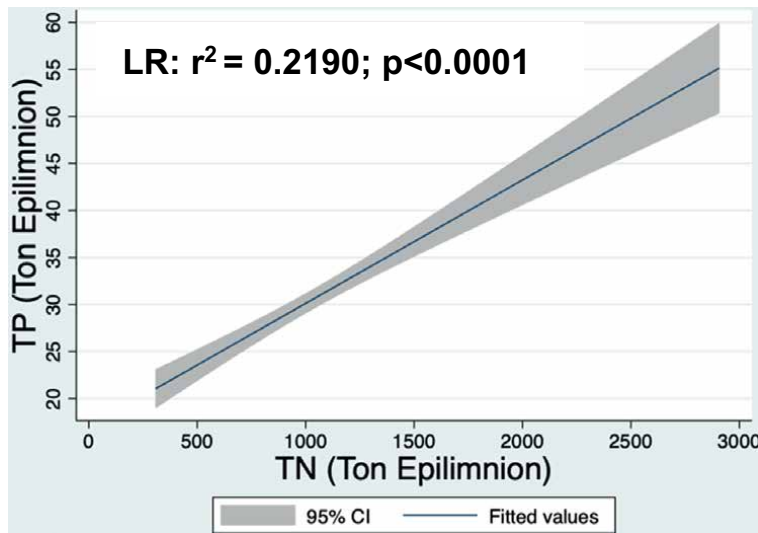


Figure 5. Linear regression (with 95% confidence limit) plot between monthly means of TN and TP epilimnetic loads during 1969–2001.

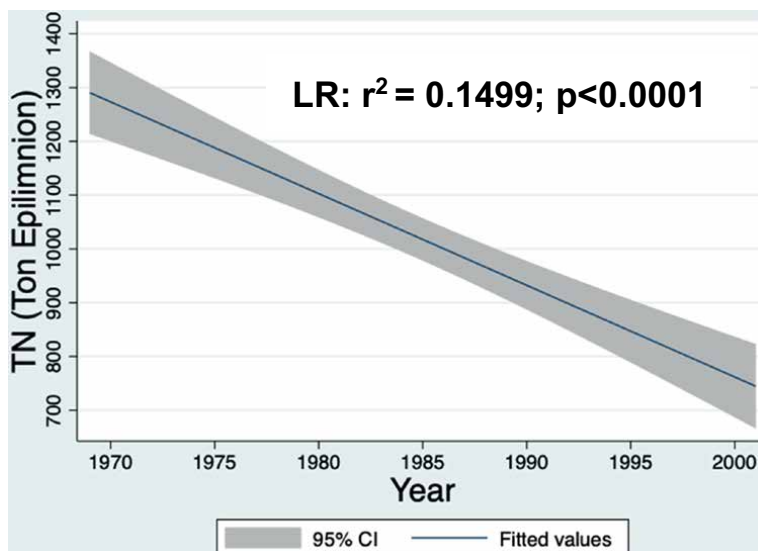


Figure 6. Temporal (1969–2001) changes in the load of total nitrogen (TN): Linear prediction (with 95% confidence limit) and regression between annual means and years.

the temporal sequence record of the mass ratio between those nutrients resulted in a declining trend. **Figure 5** represents, probably, a misleading, distorted conclusion, which is corrected by the mass ratio time series record. The amendment is an ecological improvement that is beneficial for water quality management design.

Results given in **Figures 6–8** indicate that the nitrogen decline in the Kinneret epilimnion during 1969–2001 contributes a dominant impact on the TN/TP ratio changes.

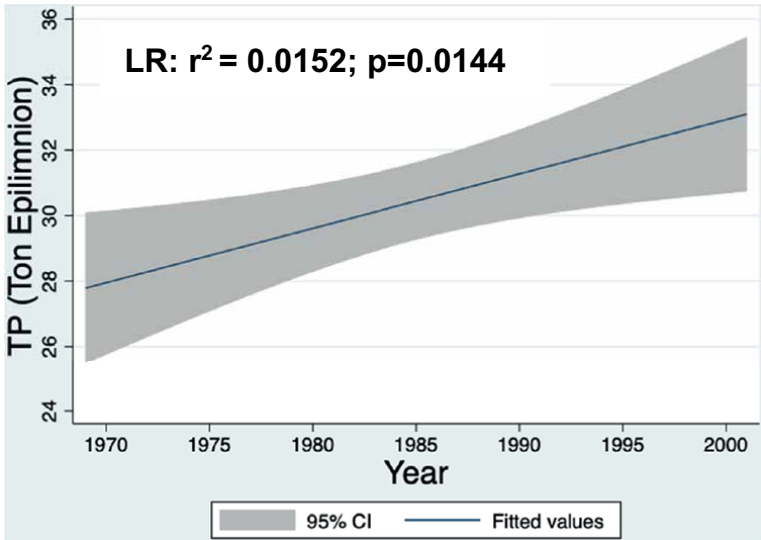


Figure 7.
Temporal (1969–2001) changes in the load of total phosphorus (TP): Linear prediction (with 95% confidence limit) and regression between annual means and years.

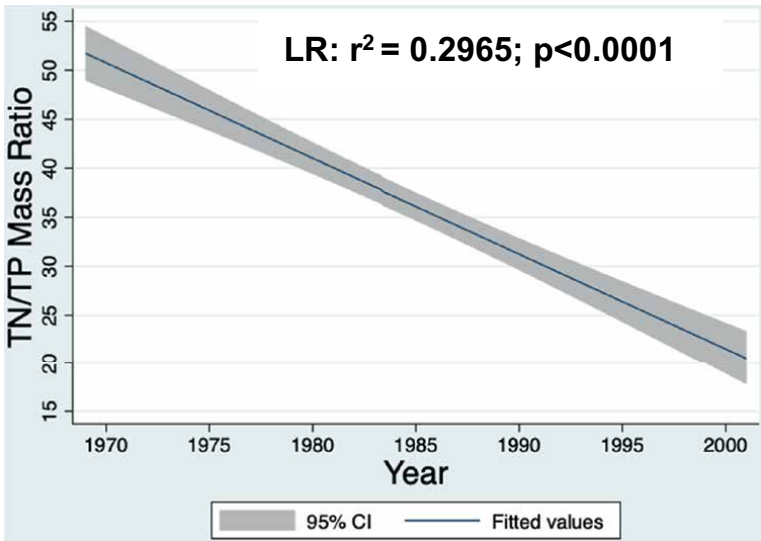


Figure 8.
Temporal (1969–2001) changes in annual (1969–2001) means of epilimnetic TN/TP, loads of TN and TP: Linear prediction (with 95% confidence limit) and regression between ratios and years.

3.3 Lake nutrients-phytoplankton relations

The data given in **Table 1** indicate the decline of algal biomass with an increasing TN/TP mass ratio. An increasing TN/TP ratio is most probable where the comprehensive ecosystem nutrient composition structure represents algal phosphorus limitation.

Results given in **Table 2** indicate phytoplankton enhancement, as well as epilimnetic TP elevation and a decline in TN loads.

The monthly data given in **Table 3** confirm a significant summer-fall decline in phytoplankton biomass and nutrient loads (except Chlorophyta), while Cyanophyta and Chlorophyta show enhancement.

Algal group	r^2	p
Total Phytoplankton	0.0416	<0.0001
Non-Pyrrhophyta	0.0342	0.0003
Cyanophyta	0.0141	0.0189
Bacillariophyta	0.0148	0.0161
Chlorophyta	0.0283	0.0008
Pyrrhophyta	0.0191	0.0063

Table 1.

Linear regression parameters (r^2 , p) of the correlations between monthly means of TN/TP ratio values and phytoplankton groups (Chlorophyta, Bacillariophyta, Cyanophyta, Pyrrhophyta, total phytoplankton, and non-Pyrrhophyta) mean epilimnetic biomass ($g(ww)/m^2$) in the Kinneret epilimnion during 1969–2001.

Nutrient/algal group	Trend	Significance	r^2	p
TP	IN	S	0.0171	$p = 0.0096$
TN	DE	S	0.1429	$p < 0.0001$
Cyanophyta	IN	S	0.0638	$p < 0.0001$
Bacillariophyta	IN	S	0.0553	$p < 0.0001$
Chlorophyta	IN	S	0.0965	$p < 0.0001$
Pyrrhophyta	DE	NS	0.0025	$p = 0.9040$

Table 2.

Linear regression parameters (r^2 , p) (S = significant; NS = not significant) of the correlations between temporal changes (IN = increase; DE = decline) (annual means) from 1969–2001 of nutrients (TN, TP; tons) and phytoplankton groups (Chlorophyta, Bacillariophyta, Cyanophyta, Pyrrhophyta) biomass ($g(ww)/m^2m^2$) in the Lake Kinneret epilimnion. The annual means of total phytoplankton, which is dominated by Pyrrhophyta, showed a low-level significant increase ($r^2 = 0.0170$; $p = 0.0098$).

Nutrient and algal group	Trend	Significance	r^2	p
TP	DE	S	0.2319	$p < 0.0001$
TN	DE	S	0.1770	$p < 0.0001$
Total Phytoplankton	DE	S	0.2088	$p < 0.0001$
Non-Pyrrhophyta	DE	S	0.0317	$p = 0.0004$
Cyanophyta	IN	S	0.0241	$p = 0.0021$
Bacillariophyta	DE	S	0.0630	$p < 0.0001$
Chlorophyta	IN	NS	0.0006	$p = 0.6267$
Pyrrhophyta	DE	S	0.1489	$p < 0.0001$

Table 3.

Linear regression parameters (r^2 , p) (S = significant; NS = not significant) of the correlations between monthly changes (IN = increase; DE = decline) (monthly means) during 1969–2001 of nutrients (TN, TP; tons) and phytoplankton group biomass (g(ww)/m²): Total phytoplankton, non-pyrrhophyta, chlorophyta, bacillariophyta, cyanophyta, and pyrrhophyta in the Lake Kinneret epilimnion.

3.4 Periodical changes of nutrient concentrations in Jordan waters

3.5 The nutrient correlation with *Peridinium* and Cyanophyta

The results given in **Figure 9** confirm a significant correlation between TN load and *Peridinium* biomass in the epilimnion of Lake Kinneret. The definition of external and internal-supported nitrogen availability as a limiting factor for *Peridinium* algal growth is therefore confirmed.

The results given in **Figure 10** confirm a significant correlation between TP load and *Peridinium* biomass in the epilimnion of Lake Kinneret. It is suggested that the TP load increase in the Kinneret epilimnion resulted from internal P-mediated proliferation of *Peridinium* cells. In other words, phosphorus is transported through

Nutrient \ Period	Period		
	1970–1985	1986–2000	2001–2018
NH ₄	0.138	0.089	0.058
NO ₃	1.619	1.932	2.021
N-org.	1.030	0.550	0.381
Kjeldhal total	1.168	0.640	0.434
Kjeldhal dissolved	0.577	0.332	0.232
TN	2.774	2.565	2.44
TP	0.236	0.176	0.117
TDP	0.045	0.040	0.033
mcm	467	427	355
TN/TP	11.75	14.57	20.85

Table 4.

Periodical (1970–1985; 1986–2000; 2001–2018) means of nutrient concentrations (ppm): Ammonium (NH₄), nitrate (NO₃), organic nitrogen (n-org.), Kjeldahl total, Kjeldahl dissolved, total nitrogen (TN), total phosphorus (TP), and TN/TP ratio (ppm/ppm) in the Jordan River (Huri Station).

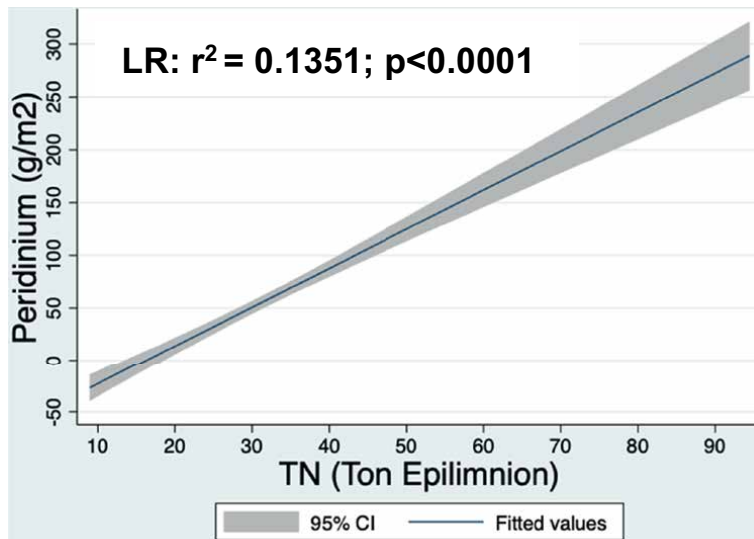


Figure 9.
 Linear prediction (with 95% confidence limit) correlative regression between monthly means of *Peridinium* spp. biomass (g(w)/m²) and epilimnetic TN load (tons) in Lake Kinneret during 1969–2001.

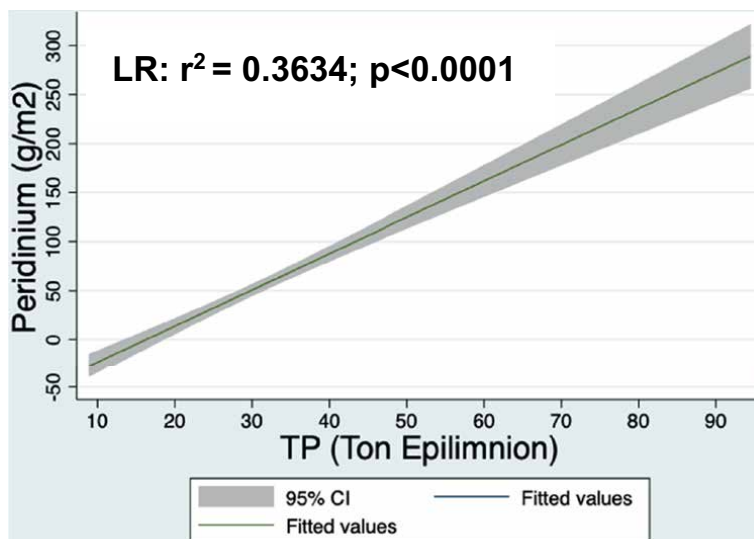


Figure 10.
 Linear prediction (with 95% confidence limit) correlative regression between monthly means of *Peridinium* spp. biomass (g(w)/m²) and epilimnetic TP load (tons) in Lake Kinneret during 1969–2001.

vegetative *Peridinium* cyst germination, migrating upwards into the light-penetrated, warm, and oxidized epilimnion layer. Among the four major phytoplankton algal groups – Chlorophyta, Bacillariophyta (diatoms), Pyrrophyta (*Peridinium* spp.), and Cyanophyta (blue-green) – only blue-greens presented significant temporal (1969–2001) changes in biomass density. Evaluated statistical correlations between grouped non-Pyrrophyte phytoplankters and TN and TP were calculated: Non-Pyrrophyte versus TN was low-level significant ($r^2 = 0.0201$; $p = 0.0058$), whilst a slightly higher

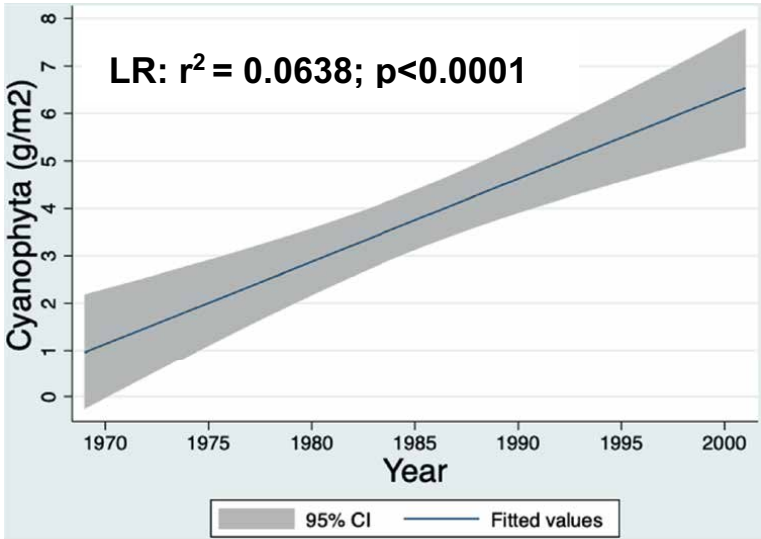


Figure 11. Temporal (1969–2001) changes tested as linear regression (with 95% confidence limit) between annual mean biomass density ($\text{g}(\text{ww})/\text{m}^2$) of cyanophyta biomass and years in Lake Kinneret.

level of significance was observed with TP: $r^2 = 0.0359$ and $p = 0.0009$. Significant correlation was indicated between TN and cyanophytes and *Peridinium*. Consequently, reduced availability of TN was followed by *Peridinium* decline, whilst Cyanophyta enhancement. It is suggested that the low-significant positive correlation found between non-Pyrrhophyta and nutrient (TN, TP) availabilities indicates algal growth enhancement, confounding the top-down cascading effect of grazing depression through the removal of unwanted zooplankton predator fish management. Nevertheless, *Peridinium* consumption by zooplankton (except for large-bodied rare rotifers, *Asplanchna spp.*) was not recorded. **Figures 11, 14.**

3.6 PCA analysis of nutrients and phytoplankton predominance

The inquiry into this issue was not emphasized by Kinneret investigators and ecosystem model constructors. In the early 1980s, scientific discoveries about

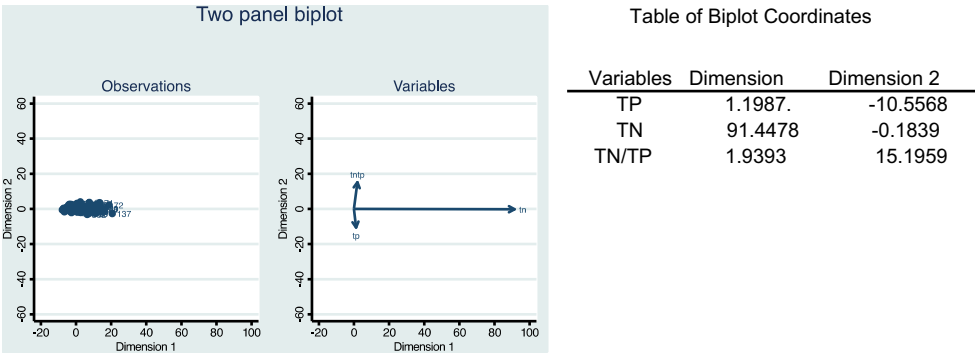


Figure 12. Biplot PCA multivariate analysis: TP, TN, and TN/TP ratio.

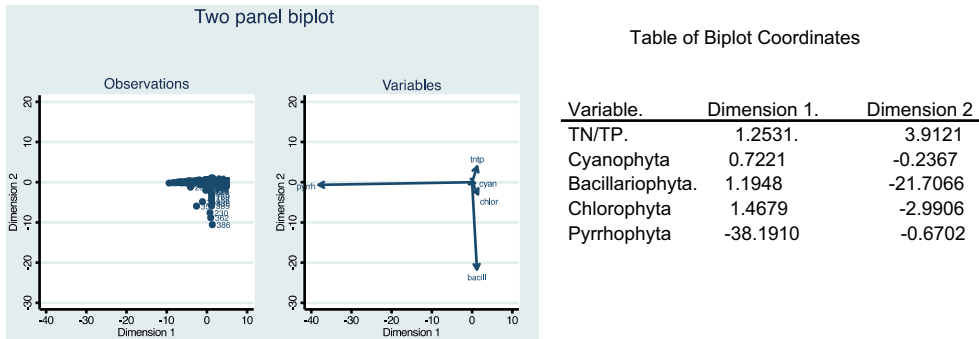


Figure 13.
Biplot PCA multivariate analysis.

nitrogen-fixing cyanophytes gained public attention. As in many other lakes in the Northern Hemisphere, predictive precaution was confirmed during the summer of 1994 in Lake Kinneret, when a bloom of *AO* was documented. A retrospective evaluation of the preliminary information about the TN/TP ratio in the Kinneret epilimnion (**Figures 5–8**) justified those preconceived warning symptoms.

Results of PCA analysis, shown in **Figure 12**, indicate that among TP, TN, and TN/TP variables, the TN is a principal component.

TN/TP Mass Ratio, Cyanophyta, Bacillariophyta, Chlorophyta, Pyrrhophyta.

Results of PCA analysis, shown in **Figure 13**, indicate that among Cyanophyta, Bacillariophyta, Chlorophyta, Pyrrhophyta, and TN/TP variables, the Pyrrhophyta (*Peridinium*) and Bacillariophyta are principal components, and the decline of Pyrrhophyta and Bacillariophyta is prominently distinct.

3.7 Long-term (1970–2018) temporal changes of nutrient concentrations in the Jordan waters

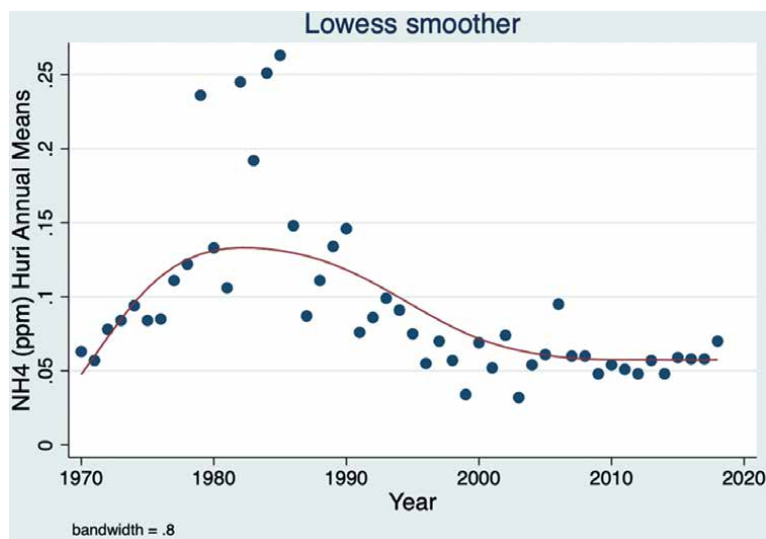


Figure 14.
Lowess smoother plot of temporal (1970–2028) changes in monthly means of NH_4 concentration (ppm) measured in the River Jordan (Huri Station).

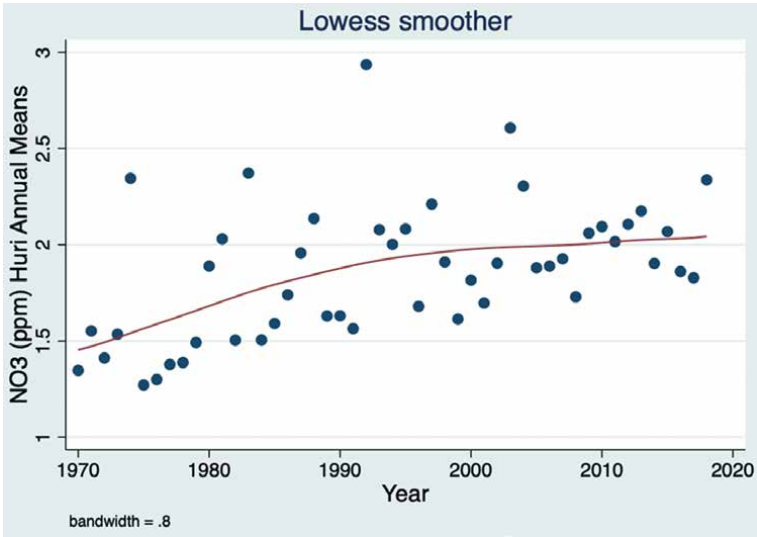


Figure 15. Lowess smoother plot of temporal (1970–2028) changes in monthly means of NO₃ concentration (ppm) measured in River Jordan (Huri Station).

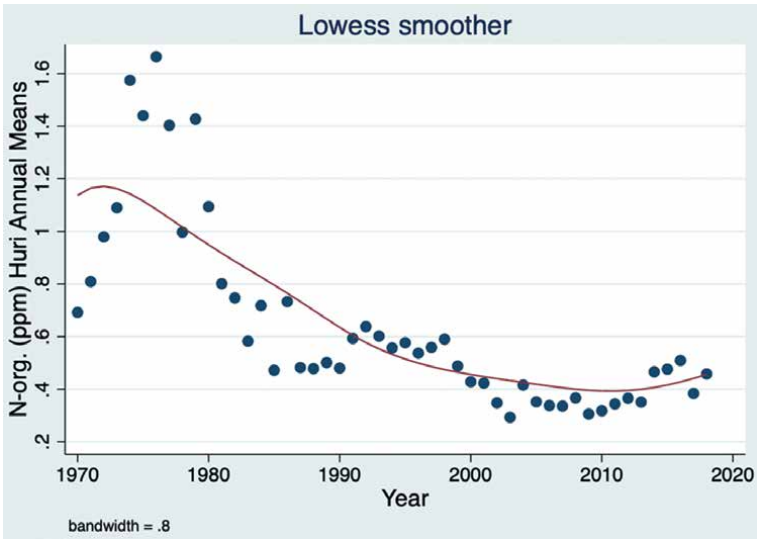


Figure 16. Lowess smoother plot of temporal (1970–2028) changes in monthly means of N-org concentration (ppm) measured in the River Jordan (Huri Station).

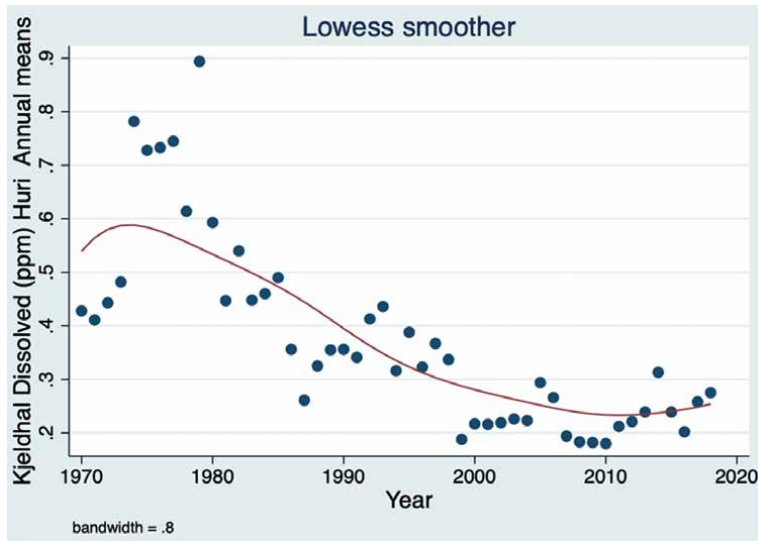


Figure 17.
 Lowess smoother plot of temporal (1970–2028) changes in monthly means of Kjeldahl dissolved concentration (ppm) measured in River Jordan (Huri Station).

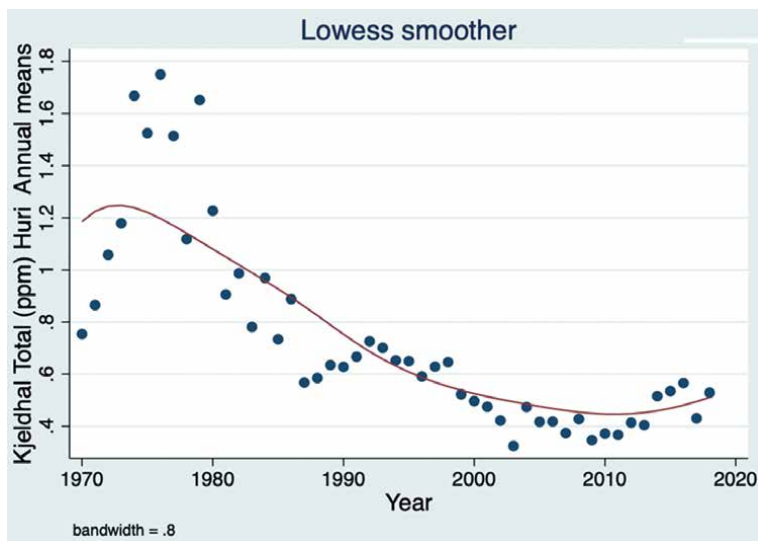


Figure 18.
 Lowess smoother plot of temporal (1970–2028) changes in monthly means of Kjeldahl total concentration (ppm) measured in the River Jordan (Huri station).

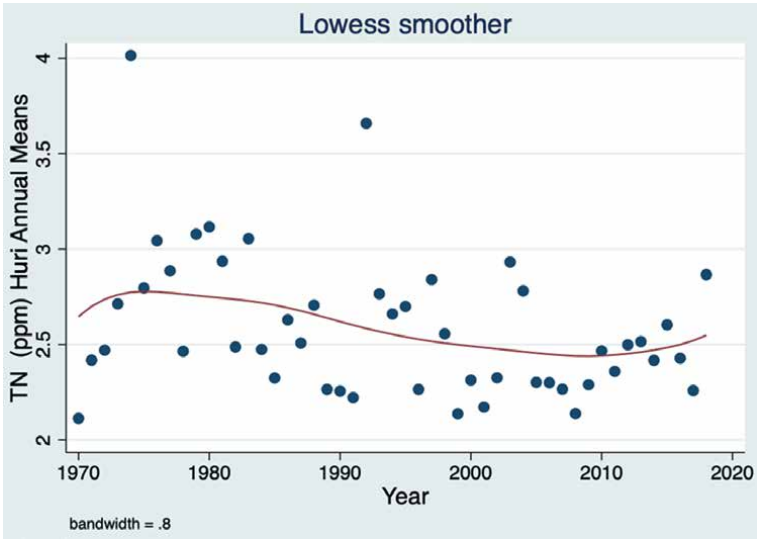


Figure 19. Lowess smoother plot of temporal (1970–2028) changes in monthly means of TN concentration (ppm) measured in River Jordan (Huri station).

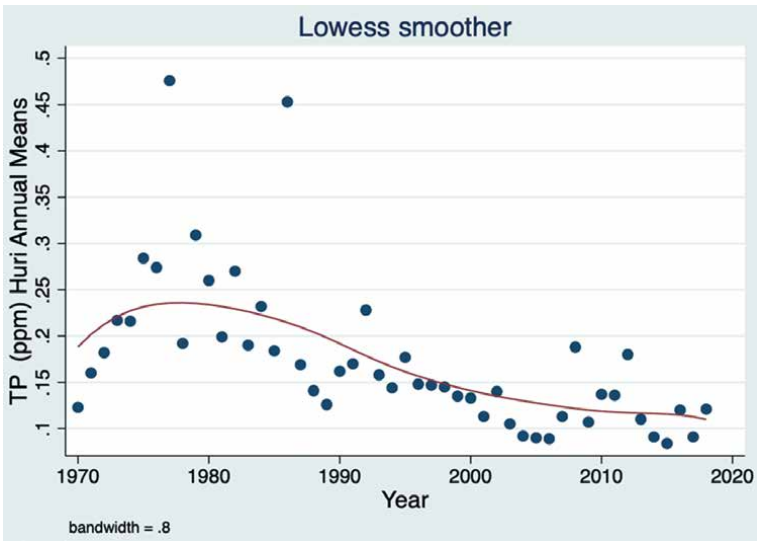


Figure 20. Lowess smoother plot of temporal (1970–2028) changes in the means of TP concentration (ppm) measured in the River Jordan (Huri Station).

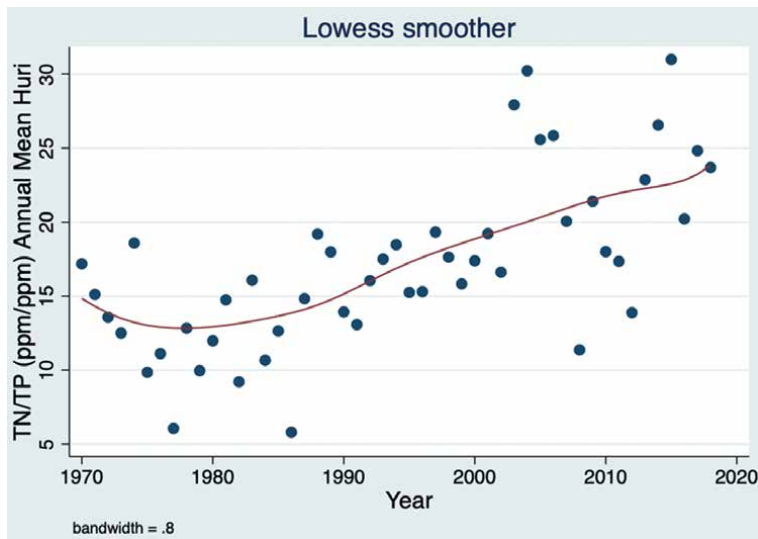


Figure 21.
 Lowess smoother plot of temporal (1970–2028) changes in the means of TN/TP ratio concentration measured in River Jordan (Huri station).

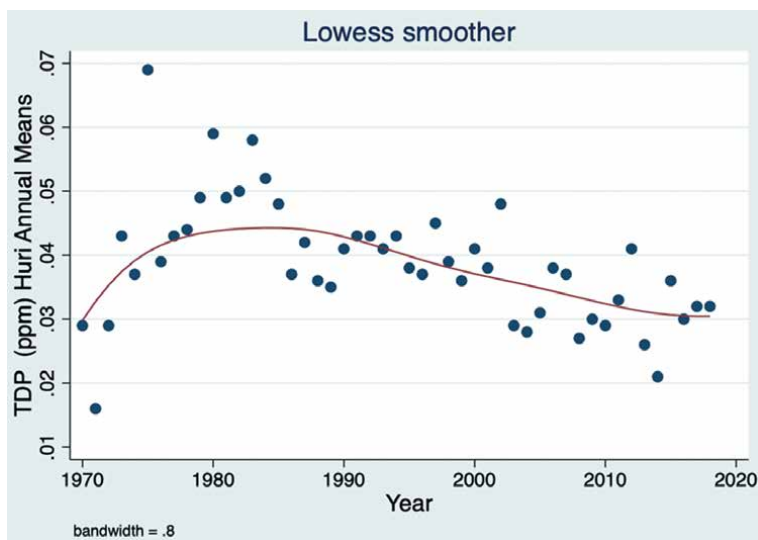


Figure 22.
 Lowess smoother plot of temporal (1970–2028) changes in monthly means of TDP concentration (ppm) measured in River Jordan (Huri Station).

4. Discussion

Terrestrial and aquatic, particularly in Lake Kinneret, cyanobacteria are widely distributed organisms in Israel [27–32]. Despite a continuous long-term record of Cyanophyta, in recent decades, it has not been a source of substantial concern for drinking-water quality. This is because of their low biomass density and species

diversity, which excluded toxic N₂-fixers, while random blooms of *Microcystis* spp. occurred in reservoirs and fishponds; no other cyanobacteria were recorded. The recorded short-term *Microcystis* blooms in Lake Kinneret were a response to Jordan River floods during early winter, which imported nitrate loads prior to the *Peridinium* blooming season [33]. Ecological sorting of cyanobacteria by properties includes, among others, single-cell or colonial, filamentous or round-shaped colonial types, toxic and non-toxic species, N₂-fixers and non-fixers, buoyancy capabilities, and vulnerability to consumer herbivory, and most crucially, response to nutrient regimes and composition. Each one of these single sorting types is relevant to the threatening level of water quality deterioration. Nevertheless, the N₂-fixing capabilities and toxicity of *AO* and *Cylindrospermopsis raciborskii* have initiated crucial concerns among water supply managers regarding the water quality of Lake Kinneret.

Suitable conditions for cyanobacteria, which gradually developed in Lake Kinneret during the 1980s and 1990s, enabled the decline of *Peridinium* and encouraged cyanobacteria enhancement. Furthermore, the development of nitrogen deficiency, accompanied by phosphorus sufficiency, significantly affected certain species, particularly HFCB such as *AO*. The most noticeable change in the Kinneret ecosystem was the modification of the nitrogen and phosphorus input loads and, consequently, their epilimnetic availabilities. Before the Hula drainage, a major part of the external nitrogen supply was in the form of ammonium. The algal affinity for this form of nitrogen is high and was favored by the bloom-forming *Peridinium*. After the Hula drainage, the external supply of ammonium was drastically reduced, and the dominant nitrogen form became nitrate. However, the prompt implementation of agricultural management was achieved through the establishment of 1,700 hectares of fishponds. The typically low winter temperatures required continuous headwater flows through the aquaculture ponds, resulting in external nutrient-mediated loads of high-quality headwater being introduced into Lake Kinneret. Furthermore, heavy nutrient loads mediated by domestic and dairy raw sewage effluents were supplied to the lake. Consequently, sewage and fishpond effluents replaced the pre-Hula drainage conditions that were advantageous for ammonium. Radical and far-reaching management measures were carried out, including sewage removal and fishpond restrictions, with an 80% conversion to field crops [21]. Despite nitrate migration from the oxidized peat soil, organic nitrogen input was reduced, and nutrient conditions in the Kinneret epilimnion were modified [28]: The N/P mass ratio declined, phosphorus replaced nitrogen as the limiting factor, and cyanobacteria replaced *Peridinium* dominance. It soon became clear that the outbreak of *AO* in the summer of 1994 and the subsequent HFCB blooms were not one-time phenomena. Future predictions of repeatable outbreaks of HFCB had far-reaching consequences for the design of Lake Kinneret's management strategies.

It is widely known that the removal or reduction of external nutrient inputs reduces or prevents lake eutrophication [34]. However, the exceptional case of the Kinneret ecosystem confirmed that external nitrogen removal without a simultaneous decline in phosphorus prevented eutrophication in the usual common sense, but water quality deteriorated through HFCB enhancement. The Kinneret ecosystem has far-reaching potential for maintenance against outbreaks of toxic and non-toxic, N₂-fixing and non-N₂-fixing cyanobacteria. Management design aimed at ecosystem improvement includes phosphorus reduction by enhancement of summer

irrigation in the Hula Valley and renewal of the hydrological system structure aimed at bypass design for the diversion of peat soil rainwater directly toward the lake. This is because there is a nitrogen decline (denitrification and sedimentation) within the LAH ecosystem.

The outbreak of AO was recorded at 2,500 and 1,000 filaments/cm³ during the summers of 1994 and 1995, respectively [12]. From 1994 onwards, toxic N₂-fixing cyanobacteria (HFCB) were sporadically recorded. Fluctuating densities of the toxic, non-N₂-fixing *Microcystis* spp. colonies, spread over most of the lake surface, were recorded every year. The existence of microcapsules within the AO cells enables buoyancy. The altitude of the National Water Carrier (NWC) intake is 215 meters below sea level. From there, water is pushed upward to 5 meters above sea level, that is, an altitude difference of 220 meters along a horizontal distance of about 30 meters – resulting in a 20-atmosphere pressure difference. Though the created pressure difference induces the collapsing of the intracellular capsules and cancellation of the buoyancy capabilities, enabling removal through sedimentation traps [35], toxicity remains despite buoyancy cancellation. The level of *Microcystins* toxicity is routinely sampled at 7–15 stations throughout the entire lake surface area, contributed by *Microcystis* spp. bloomed aggregation is monitored. Results indicate concentrations below the permitted level in the NWC intake lake zone. The herbivore zooplankton gut content was studied [1], and a low level of consumption of cyanobacteria, along with the predominance of Chlorophyta and Bacillariophyta, was confirmed.

Prior to the record of AO in 1994, more than 40 Cyanophyte species were documented in Lake Kinneret [27]. Of these, only one genus (*Microcystis* spp.) is HFCB but is a non-N₂-fixer. Since the late 1960s, the Lake Kinneret ecosystem has undergone significant external and internal environmental changes: Shifting from phosphorus to nitrogen as the limiting nutrient, which led to the replacement of *Peridinium* spp. by Cyanophyta-dominated blooms. The predictive capabilities of earlier ecosystem models [1, 2] were evaluated retrospectively.

Since the early 1930s, three critical management achievements were implemented within the lake and its drainage basin ecosystems: The construction of the south dam (1933), which enabled human control of Lake Water Level Fluctuation (WLF); the construction of the NWC (1964), which established Lake Kinneret as a national source of domestic water supply through a daily withdrawal of 10⁶ m³; and the Hula drainage (1957), which enhanced nitrate migration into the lake. The commonly accepted limnological paradigm of lake and reservoir ontogeny suggests that water-level fluctuations may increase sedimentary nutrient release, causing long-term eutrophication [36, 37]. Consequently, eutrophication is attributed to the enhancement of nutrient loads. After the Hula drainage was completed and nitrate migration was enhanced, national concern about Kinneret eutrophication increased. A distinct decline in nitrogen (**Figure 6**) and a slight increase in phosphorus loads (**Figure 7**) in the epilimnion were documented. Taking into account nitrogen availability as the leading factor, water quality in Lake Kinneret improved, while, due to the long-term minor elevation of phosphorus and the N/P mass ratio decrease (**Figure 8**), water quality deteriorated. The desired algal community composition, dominated by *Peridinium* spp. biomass in Lake Kinneret indicates better water quality [36], whereas the enhancement of Cyanophyta, including HFCB (*Aphanizomenon* spp. and *Cylindrospermopsis* spp.), indicates water quality deterioration. The ecosystem-modulated evaluation of the Kinneret ecosystem [1, 2, 36, 37] did not indicate model predictions of optional developments

suitable for the enhancement of cyanobacterial biomass. None of these documents formulated a clear, distinct precaution or an optional predominant alarm signal emphasizing the replacement of *Peridinium* by Cyanophyta in Lake Kinneret. These scientifically modulated or “state-of-the-art” brilliant evaluations are more ecolimnological “Post Mortem Autopsy” than valuable management predictions. Despite the predicted management design of unwanted fish removal, it was concluded as a crossword clue [1, 37].

The long-term background of nutrient dynamics, which created those ecosystem modifications, was widely, partially, and comprehensively documented. Several models were suggested [1, 2, 37], whereas none of them presented a prediction of the management design of the watershed nutrient regime resulting from respective modulated nutrient dynamics. Furthermore, forecasted optional modifications of nutrient availabilities that support the enhancement of cyanobacterial blooms are not considered as thoroughly as they are for zooplankton.

Model A [1] considered two types of phytoplankton communities that characterized different eco-limnological conditions: A) Low density of Pyrrhophyta accompanied by enhanced biomass of non-Pyrrhophyte tiny algae components, mostly Chlorophyta, Bacillariophyta, and nontoxic non-colonial nanoplanktonic Cyanophyta. B) The commonly recorded late-winter-spring-early-summer heavy bloom of *Peridinium gatunense* and low density of nanophytoplankton. In both cases, the summer phytoplankton assemblages are dominated by nanophytoplankton, of which Cyanophyta biomass is low. The *Peridinium* bloom is characterized by high availability of nitrogen (mostly as nitrate), starting shortly before bloom initiation, resulting from heavy external inputs. Independently, the biomass enhancement of *Peridinium* after bottom excystation and the pelagic high rate of vegetative cell multiplication is also abruptly supported by external and internal epilimnetic phosphorus enrichment elevation. Four channels of phosphorus input into the Kinneret epilimnion are indicated: External aquatic headwaters, atmospheric dust deposition, internal flux from bottom sediments through mediated *Peridinium* cells, and geochemical-bacteriological processes. As soon as the nitrogen supply is overwhelmed, the *Peridinium* creates a flourishing bloom, which, by itself, is a consequent enhancement of phosphorus input into the epilimnion. Nevertheless, from the late 1980s to early 1990s, external aquatic input of nitrogen diminished, but bottom phosphorus supply did not decline, supporting epilimnetic nutrient availability conditions. Though the epilimnetic nutrient balance was modified into “Sufficient Phosphorus and Insufficient Nitrogen,” which is preferably favored by cyanobacteria and unfavored by *Peridinium*. The interaction between nutrient composition, such as the mass ratio between the availabilities of nitrogen and phosphorus, was not considered as a management factor in previous studies [1, 2, 36, 37]. The appropriate evaluation of the ecological processes that supported a significant change in the algal community includes long-term analysis. Statistical analysis of long-term precipitation (mm/year; number of rainy days/year) records (Dafna Station, 1969–2001) indicates no significant fluctuations. Consequently, other environmental factors within the ecosystem, such as anthropogenic management changes in the Hula Valley, which affected the nutrient input regime (**Figure 7**), were investigated. Consequently, temporal TN and TP dynamic fluctuations are dissimilar due to the different management impacts on TN and TP migration from the Hula Valley, as well as unequal rates of internal processes resulting in changes in the variance of the mass ratio between them (**Figure 8**).

4.1 The uniqueness of AO blooming in 1994

The epilimnetic section (0–15 m) of the Kinneret ecosystem during summer time is similar to a bio-chemostat ecosystem: High temperature leading to metabolic range demands enhancement of all living organisms from planktonic algae and bacteria through protozoans to metazoan invertebrates (zooplankters) and vertebrates (fish). On the other hand, headwater water and nutrient inputs and consequently their epilimnetic availabilities, strictly diminished. During summer months (August–October), the epilimnetic concentration of inorganic nitrogen ranged between 100–50 $\mu\text{g/l}$. In lakes where the concentration of inorganic nitrogen is lower than 50 $\mu\text{g/l}$, the N_2 -fixing cyanobacterium is very common [15, 16, 38]. Moreover, as a result of summer stable stratification, internal upward nutrient transfer from the hypolimnion to the epilimnion is limited despite of a high velocity daily west wind regime and significant motions of water mass. The ecological situation of limited food resources, accompanied by the enhancement of energy requirement, creates a sensitive seasonal ecosystem of the Kinneret epilimnion. Which require at least conditions supply of the following: Insufficient nitrogen, sufficient phosphorus (INSP), resulting in a lower N/P ratio, and appropriate inoculum. Nitrogen limitation interferes with non- N_2 fixing algal metabolic activity; sufficient phosphorus is required by the entire algal assemblage, including nitrogen fixers. The minimum sufficiency inoculum demand is due to the short period for the appropriate combined conditions to exist. The point-specific Kinneret summer conditions competitive advantage of the N_2 -fixer cyanobacterium is their ability to utilize atmospheric nitrogen by the nitrogenase enzyme. Despite the short-term suitable niche for AO bloom formation during summer 1994, the long-term perspective confirms the N/P mass ratio and INSP availability for cyanobacteria and HFCB, including enhancement and consequently replacing *Peridinium* dominance unless epilimnetic nitrogen load is significantly enriched. During the 15 years prior to the AO bloom outbreak, epilimnetic nitrogen deficiency was progressively developed (**Figure 6**). Wind velocity record indicates exceptionally low regimes during the summers of 1994 and 1995. It is a factor of mixing water mass reduction, which diminished the upward transfer of inorganic nitrogen as well. The long-term decline of nitrogen, whilst phosphorus was enhanced, resulting N/P mass ratio decrease and the AO outbreak were early warning signals of the cyanobacterial threat to water quality. The *Peridinium* decline and blue-green replacement, which came later, confirmed this trend, but not necessarily eutrophication and dissolved oxygen depletion. Moreover, the proliferation of nitrogen fixers contributes enrichment of nitrogen load (supporting the renewal of *Peridinium* blooming) as already measured during the AO summer bloom.

Results given in **Table 4** and **Figures 15–22** emphasize the role of sewage removal, fishpond restriction, and replacement by field crops in the achievements of the post-drainage Hula Valley on nitrogen and phosphorus migration. These management operations highlight the potential role that nitrogen and phosphorus input loads play in cyanobacteria dynamics. Nitrogen forms supplied from the Hula wetlands to the Kinneret ecosystem before 1957 were mostly ammonia and organic nitrogen [22]. The Hula drainage and the consequent peat soil oxidation process caused a shift in the migrated nitrogen composition from ammonia to a nitrate-dominated content. A major part of the drained Hula soils (peat) was organic-nitrate-rich and weakly bound to the soil particles. Practically, the upper soil layers of the Hula post-drainage were created as a huge bulk of easily flushed inorganic nitrogen – nitrate [25]. Unlike the anthropogenic impact on the decline of organic

nitrogen and phosphorus, nitrate migration, and consequently TN (**Figures 6, 19**) is mostly affected by soil moisture contributed by rainfall and summer irrigation capacities. Therefore, a gradual temporal enhancement of nitrate concentration in the Jordan waters was recorded (**Figure 15**). The decline in phosphorus concentration in the Jordan waters, supported by management (**Figures 7, 20**) combined with nitrate enhancement, affected the TN/TP ratio elevation (**Figures 8, 21**). Despite the decline in external phosphorus supply, an increase in epilimnetic concentration was recorded during the mid-1990s, supported by internal sources [11, 12]. Because nitrate is the dominant nitrogenic-component within the Jordan waters, the TN concentration did not decline (**Figure 19**). The affinity of ammonia within the metabolic requirements of *Peridinium* is high, resulting, probably, in its heavy bloom existence before drainage and shortly (mid-1990s) later when cyanobacteria enhanced. Despite peat soil oxidation and nitrate migration enhancement (**Figure 15**), ammonia and organic nitrogen concentrations were severely enhanced as a result of sewage and fishpond effluents (1970–1985: **Table 4, Figures 14, 16–18**). Sewage and fishpond effluent inflows into the Jordan discharge also enhanced the migration of phosphorus (**Figures 20, 22; Table 4**). The enhancement of organic nitrogen, ammonia, and phosphorus loads (1970–1985) through sewage and fishpond effluents (1970–1985) was later (1986–2018) declined, excluding nitrate. Consequently, total nitrogen concentration in the Jordan waters was fairly stable (**Figure 19**) whereas a significant decline in organic nitrogen and ammonia occurred as a result of sewage and fishpond effluent management removal. The *Peridinium* decline and cyanobacteria enhancement were initiated during the mid-1990s despite nitrate availability, taking into account the higher affinity of organic nitrogen and ammonia to *Peridinium* growth than nitrate. With respect to external Kinneret inputs of nitrogen and phosphorus, shortly after drainage (1970–1986), conditions achieved *Peridinium* dominance, whilst later on cyanobacteria were favored: Nitrogenic forms (organic and ammonia) deficiency and phosphorus (internal and external) sufficiency. The enhancement of the TN/TP ratio (**Table 4, Figure 21**) is due to the enhancement of nitrate and consequently TN, whilst the cyanobacteria-preferred nitrogen forms (NH₄, organic – N) declined. The decline of the TN/TP mass ratio, resulting from nitrogen deficiency and phosphorus sufficiency in the epilimnion of Lake Kinneret, is widely documented [11, 15, 16, 21] and enhanced N₂-fixing cyanobacterium. The intra cellular enabled nitrogen-fixer AO to flourish during the summer of 1994 in Lake Kinneret.

5. Conclusive summary

The seasonal respective response of nutrient input with Jordan discharge is shown in **Figures 1–4**. The dependence of epilimnetic loads of nitrogen and phosphorus on the external inputs is emphasized in **Figures 5–8**. Further consideration implies the resulting cyanobacteria enhancement and *Peridinium* decline, reinforced by the PCA analysis (**Figures 9–13**). Justification of the short-term (1970–2001) changes' similarity to the long-term (1970–2018) period is confirmed in **Figures 14 and 16–22**. The significant impact of anthropogenic involvement within the Hula Valley's ecological structure is limited to sewage removal and the conversion of fishponds to field crops. On the other hand, the Hula drainage caused peat soil oxidation and modification of migrated nitrogen from ammonia before drainage to nitrate after

drainage. The very high peat nitrogen content and mobility through wettability imply its continuous temporal increase (**Figure 15**). The focused concept of the paper is aimed at the management implementation within the Hula Valley after drainage.

During the late 1960s to 1990s, regular heavy late winter-spring-early summer blooms of *Peridinium* were recorded. Then, *Peridinium* densities declined, and cyanobacteria increased. The evaluated study of the long-term (1969–2001) spatio-temporal record of hydrological dynamics, combined with nutrient migrations from the Kinneret drainage basin and phytoplankton and nutrient distributions in Lake Kinneret, confirmed interactions between them. The data shown in **Figures 1–5** confirm that, in correlation with seasonal rainfall distribution, the epilimnetic loads of TP and TN are high in winter and decline in summer. The temporal data (1969–2001) (**Figures 6–8**) confirm a decline in TN and a slight increase in TP, and therefore, the TN/TP mass ratio in the epilimnion of Lake Kinneret declined. Low ratios of N/P are well-known as suitable conditions for Cyanophyta enhancement [15, 16]. The merit of nitrogen availability to *Peridinium* growth and multiplication is shown in **Figure 9**: the more nitrogen is available, the heavier the *Peridinium* biomass density. However, *Peridinium* biomass density also increases with the availability of TP in the epilimnion (**Figure 10**). The concluded outcome about nitrogen deficiency and phosphorus sufficiency as N₂-fixer cyanobacterium enhancement (**Figures 9, 10**) is therefore unlikely, whilst it is likely if TP and TN sources are indicated. The sources of the epilimnetic nitrogen are mostly external, through infrastructure, rocky basement erosion by headwater flows, and, to a lesser extent, through internal ammonium oxidation during stratification and destratification and N₂ fixation. Temporal stability and a slight increase in TP external supply through erosion and dust deposition were documented. Moreover, a significant part of epilimnetic phosphorus import is carried out by the vegetative, multiplied bloom-forming *Peridinium*. A significant part of this transported phosphorus is due to previously incorporated phosphorus from the sediments by the settled *Peridinium* cysts into the sediments after cystation. During late winter and excystation occurrence, vegetative cells acquire motile ability by flagellum and migrate into the epilimnion, creating bloom onset by the high rate of cell division. In early summer (June–July), cell degradation is enhanced. Throughout the entire bloom existence, and particularly during bloom collapse, the release of inorganic dissolved phosphorus (DIN) into the epilimnion was documented [1, 11, 12, 27]. Conclusively, epilimnetic nitrogen decline resulted from external supply reduction and internal phosphorus flux enhancement by *Peridinium* cell transport, creating suitable conditions for a particular AO outbreak [10–12, 35]. Long-term depletion of nitrogen external supply, accompanied by stability of phosphorus inputs, predicts enhancement of cyanobacteria biomass at the expense of *Peridinium*. The outcome for future management is the reduction of external phosphorus input. Enhancement of external nitrogen input, as nitrate, might be implemented through an irrigation regime.

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Author contribution

The author directed data evaluation, presentation design, computerization, and the preparation of the original and final draft versions.

Data availability statement

The data presented in this study is available upon request from the corresponding author.

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Cyanobacteria are photosynthetic microorganisms of fundamental importance to Earth's ecosystems and to a wide range of biological and applied studies. Their roles in oxygenic photosynthesis, nutrient cycling, and biomass production have made them a central focus of research in energy-related processes, environmental systems, and industrial biology. *Cyanobacteria – Exploring Their Role in Energy, Environment, and Industry* provides an overview of key concepts and current research related to cyanobacterial biology and applications. The volume covers fundamental aspects of photosynthesis, including the structural and functional roles of Photosystem II cofactors, as well as advances in genetic modification, cyanobacteria as biological chassis, algae-based biorefineries, wastewater treatment, and the ecological dynamics that influence cyanobacterial populations in natural environments. Rather than emphasizing technological deployment, this book focuses on foundational knowledge and representative studies that illustrate the diversity and relevance of cyanobacteria across scientific disciplines. It is intended as a reference for researchers, graduate students, and professionals seeking a clear and comprehensive introduction to the field.

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